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I, KIM MARSHALL, MANAGER EXAMINATION SUPPORT AND SALES, hereby certify that the annexed is a true copy of the Provisional specification in connection with Application No. PP 2499 for a patent by AG-GENE AUSTRALIA LTD and STATE OF QUEENSLAND THROUGH ITS DEPARTMENT OF PRIMARY INDUSTRIES filed on 20 March 1998.

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

WITNESS my hand this Fourteenth day of April 1999

KIM MARSHALL
MANAGER EXAMINATION SUPPORT AND SALES

Regulation 3.2

Ag-Gene Australia Ltd

AND

State of Queensland through its Department of Primary Industries

A U S T R A L I A

Patents Act 1990

PROVISIONAL SPECIFICATION

for the invention entitled:

"Gene Expression I"

The invention is described in the following statement:

GENE EXPRESSION I

FIELD OF THE INVENTION

The present invention relates generally to a method of modifying gene expression and reagents therefor. More particularly, the present invention utilises recombinant DNA technology to post-transcriptionally modify or modulate the expression of a target gene in a cell, tissue, organ or whole organism, thereby producing novel phenotypes.

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

As used herein, the term "derived from" shall be taken to indicate that a particular integer or group of integers has originated from the species specified, but has not necessarily been obtained directly from the specified source.

BACKGROUND TO THE INVENTION

Controlling metabolic pathways in eukaryotic organisms is desirable for the purposes of producing novel traits therein or introducing novel traits into a particular cell, tissue or organ of said organism. Whilst recombinant DNA technology has provided significant progress in an understanding of the mechanisms regulating eukaryotic gene expression, much less progress has been made in the actual manipulation of gene expression to produce novel traits. Moreover, there are only limited means by which human intervention may lead to a modulation of the level of eukaryotic gene expression.

One approach to repressing, delaying or otherwise reducing gene expression utilise a mRNA molecule which is transcribed from the complementary strand of a nuclear gene to that which is normally transcribed and capable of being translated into a polypeptide. Although the precise mechanism involved in this approach is not established, it has been postulated that a double-stranded mRNA may form by base pairing between the complementary nucleotide sequences, to produce a complex which is translated at low efficiency and/or degraded by intracellular ribonuclease enzymes prior to being translated.

Alternatively, the expression of an endogenous gene in a cell, tissue or organ may be suppressed when one or more copies of said gene, or one or more copies of a substantially similar gene are introduced into the cell. Whilst the mechanism involved in this phenomenon has not been established and appears to be involve mechanistically heterogeneous processes. For example, this approach has been postulated to involve transcriptional repression, in which case somatically-heritable repressed states of chromatin are formed or alternatively, a post-transcriptional silencing wherein transcription initiation occurs normally but the RNA products of the co-suppressed genes are subsequently eliminated.

The efficiency of both of these approaches in targeting the expression of specific genes is very low and highly variable results are usually obtained. Inconsistent results are obtained using different regions of genes, for example 5'- untranslated regions, 3'-untranslated regions, coding regions or intron sequences to target gene expression. Accordingly, there currently exists no consensus as to the nature of genetic sequences which provide the most efficient means for repressing, delaying or otherwise reducing gene expression using existing technologies. Moreover, such a high degree of variation exists between generations such that it is not possible to predict the level of repression of a specific gene in the progeny of an organism in which gene expression was markedly modified.

Recently, Dorer and Henikoff (1994) demonstrated the silencing of tandemly repeated gene copies in the *Drosophila* genome and the transcriptional repression of dispersed *Drosophila*

Adh genes by *Polycomb* genes (i.e. the *Pc-G* system; Pal-Bhadra *et al*, 1997). However, such silencing of tandemly repeated gene copies is of little utility in an attempt to manipulate gene expression in an animal cell by recombinant means, wherein the sequences capable of targeting the expression of a particular gene are introduced at dispersed locations in the genome, absent the combination of this approach with gene-targeting technology. Whilst theoretically possible, such combinations would be expected to work at only low-efficiency, based upon the low efficiency of gene-targeting approaches used in isolation and further, would require complicated vector systems. Additionally, the utilisation of transcriptional repression, such as the *Drosophila Pc-G* system, would appear to require some knowledge of the regulatory mechanisms capable of modulating the expression of any specific target gene and, as a consequence, would be difficult to implement in practice as a general technology for repressing, delaying or reducing gene expression in animal cells.

The poor understanding of the mechanisms involved in these phenomena has meant that there have been few improvements in technologies for modulating the level of gene expression , in particular technologies for delaying, repressing or otherwise reducing the expression of specific genes using recombinant DNA technology. Furthermore, as a consequence of the unpredictability of these approaches, there is currently no commercially-viable means for modulating the level of expression of a specific gene in a eukaryotic or prokaryotic organism.

Thus, there exists a need for improved methods of modulating gene expression, in particular repressing, delaying or otherwise reducing gene expression in animal cells for the purpose of introducing novel phenotypic traits thereto. In particular, these methods should provide general means for phenotypic modification, without the necessity for performing concomitant gene-targeting approaches.

SUMMARY OF THE INVENTION

The invention is based in part on the surprising discovery by the inventors that cells which exhibit one or more desired traits can be produced and selected from transformed cells comprising a nucleic acid molecule operably linked to a promoter, wherein the transcription

product of the nucleic acid molecule comprises a nucleotide sequence which is substantially identical to the nucleotide sequence of a transcript of an endogenous or non-endogenous target gene, the expression of which is intended to be modulated. The transformed cells are regenerated into whole tissues, organs or organisms capable of exhibiting novel traits, in particular virus resistance and modified expression of endogenous genes.

Accordingly, the present invention provides a method of modulating the expression of a target gene in an animal cell, tissue or organ, said method at least comprising the step of introducing to said cell, tissue or organ one or more dispersed nucleic acid molecules or foreign nucleic acid molecules comprising a nucleotide sequence which is substantially identical to the nucleotide sequence of said target gene or a region thereof or complementary thereto for a time and under conditions sufficient for translation of the mRNA product of said target gene to be modified, subject to the proviso that the transcription of said mRNA product is not exclusively repressed or reduced.

In a particularly preferred embodiment, the dispersed nucleic acid molecules or foreign nucleic acid molecules comprises a nucleotide sequence which encodes an mRNA molecule which is substantially identical to the mRNA product of the target gene.

In a more particularly preferred embodiment, the dispersed nucleic acid molecule or foreign nucleic acid molecule is in an expressible form such that it is at least capable of being transcribed to produce mRNA.

The target gene may be a gene which is endogenous to the animal cell or alternatively, a foreign gene such as a viral or foreign genetic sequence, amongst others. Preferably, the target gene is a viral genetic sequence.

The invention is particularly useful in the modulation of eukaryotic gene expression, in particular the modulation of human or animal gene expression and even more particularly in the modulation of expression of genes derived from vertebrate and invertebrate animals, such

as insects, aquatic animals (eg. fish, shellfish, molluscs, crustaceans such as crabs, lobsters and prawns, avian animals and mammals, amongst others).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a copy of a diagrammatic representation of the plasmid pEGFP.BEV.1.

Figure 2 is a copy of a diagrammatic representation of the plasmid pCMV.BEV.2.

Figure 3 is a copy of a diagrammatic representation of the plasmid pCMV.VEB.

Figure 4 is a copy of a diagrammatic representation of the plasmid pCMV.BEVnt.

Figure 5 is a copy of a diagrammatic representation of the plasmid pCMV.BEVx2.

Figure 6 is a copy of a diagrammatic representation of the plasmid pCMV.BEV.VEB.

Figure 7 is a copy of a diagrammatic representation of the plasmid pCMV.BEV.GFP.VEB.

Figure 8 is a copy of a diagrammatic representation of the plasmid pCMV.BEV.SV40L-0.

Figure 9 is a copy of a diagrammatic representation of the plasmid pCMV.0.SV40L.BEV.

Figure 10 is a copy of a diagrammatic representation of the plasmid pCMV.0.SV40L.VEB.

Figure 11 is a copy of a diagrammatic representation of the plasmid pCMV.BEV.SV40L.BEV.

Figure 12 is a copy of a diagrammatic representation of the plasmid

pCMV.BEV.SV40L.VEB.

Figure 13 is a copy of a diagrammatic representation of the plasmid pCMV.SV40LR.cass.

Figure 14 is a copy of a diagrammatic representation of the plasmid pCMV.BEV.SV40LR.

Figure 15 is a copy of a diagrammatic representation of the plasmid pCMV.TYR.

Figure 16 is a copy of a diagrammatic representation of the plasmid pCMV.TYRLIB.

Figure 17 is a copy of a diagrammatic representation of the plasmid pCMVLacI.OPRSV1.GFP.TYR.

Figure 18 is a copy of a diagrammatic representation of the plasmid pCMVLacI.OPRSV1.GFP.cass.

Figure 19 is a copy of a diagrammatic representation of the plasmid pCMV.Lac.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of modulating the expression of a target gene in an animal cell, tissue or organ, said method at least comprising the step of introducing to said cell, tissue or organ one or more dispersed nucleic acid molecules or foreign nucleic acid molecules comprising a nucleotide sequence which is substantially identical to the nucleotide sequence of said target gene or a region thereof or complementary thereto for a time and

under conditions sufficient for translation of the mRNA product of said target gene to be modified, subject to the proviso that the transcription of said mRNA product is not exclusively repressed or reduced.

As used herein, the term "modulating" shall be taken to mean that expression of the target gene is either increased or reduced in amplitude or the timing of gene expression or the developmental or tissue-specific or cell-specific pattern of target gene expression is altered, compared to the expression of said gene in the absence of the inventive method described herein.

In a preferred embodiment, whilst not limiting the scope of the invention described herein, the present invention is directed to a modulation of gene expression which comprises the repression, delay or reduction in amplitude of target gene expression in a specified cell, tissue or organ of a eukaryotic organism, in particular a human or other animal and even more particularly a vertebrate and invertebrate animal, such as an insect, aquatic animal (eg. fish, shellfish, mollusc, crustacean such as a crab, lobster or prawn, an avian animal or a mammal, amongst others).

More preferably, target gene expression is completely inactivated by the dispersed nucleic acid molecules or foreign nucleic acid molecules which has been introduced to the cell, tissue or organ.

Whilst not being bound by any theory or mode of action, the reduced or eliminated expression of the target gene which results from the performance of the invention may be attributed to reduced or delayed translation of the mRNA transcription product of the target gene or alternatively, the prevention of translation of said mRNA, as a consequence of sequence-specific degradation of the mRNA transcript of the target gene by an endogenous host cell system.

It is particularly preferred that, for optimum results, sequence-specific degradation of the

mRNA transcript of the target gene occurs either prior to the time or stage when the mRNA transcript of the target gene would normally be translated or alternatively, at the same time as the mRNA transcript of the target gene would normally be translated. Accordingly, the selection of an appropriate promoter sequence to regulate expression of the introduced dispersed nucleic acid molecule or foreign nucleic acid molecule is an important consideration to optimum performance of the invention. For this reason, strong constitutive promoters or inducible promoter systems are especially preferred for use in regulating expression of the introduced dispersed nucleic acid molecules or foreign nucleic acid molecules.

The present invention clearly encompasses reduced expression wherein reduced expression of the target gene is effected by lowered transcription, subject to the proviso that a reduction in transcription is not the sole mechanism by which this occurs and said reduction in transcription is at least accompanied by reduced translation of the steady-state mRNA pool.

The target gene may be a genetic sequence which is endogenous to the animal cell or alternatively, a non-endogenous genetic sequence, such as a genetic sequence which is derived from a virus or other foreign pathogenic organism and is capable of entering a cell and using the cell's machinery following infection.

Wherein the target gene is a non-endogenous genetic sequence to the animal cell, it is desirable that the target gene encodes a function which is essential for replication or reproduction of the viral or other pathogen. In such embodiments, the present invention is particularly useful in the prophylactic and therapeutic treatment of viral infection of an animal cell or for conferring or stimulating resistance against said pathogen.

More preferably, the target gene comprises one or more nucleotide sequences of a viral pathogen of an animal cell, tissue or organ, such as but not limited to a retrovirus, for example a lentivirus such as the immunodeficiency viruses, a single-stranded (+) RNA virus such as bovine enterovirus (BEV) or Sinbis alphavirus. Alternatively, the target gene comprises one or more nucleotide sequences of a viral pathogen of an animal cell, tissue or

organ, such as but not limited to a double-stranded DNA virus such as bovine herpes virus or herpes simplex virus I (HSV I), amongst others.

With particular regard to viral pathogens, those skilled in the art are aware that virus-encoded functions may be complemented *in trans* by polypeptides encoded by the host cell. For example, the replication of the bovine herpes virus genome in the host cell may be facilitated by host cell DNA polymerases which are capable of complementing an inactivated viral DNA polymerase gene.

Accordingly, wherein the target gene is a non-endogenous genetic sequence to the animal cell, a further alternative embodiment of the invention provides for the target gene to encode a viral or foreign polypeptide which is not capable of being complemented by a host cell function, such as a virus-specific genetic sequence. Exemplary target genes according to this embodiment of the invention include, but are not limited to genes which encode virus coat proteins, uncoating proteins and RNA-dependent DNA polymerases and RNA-dependent RNA polymerases, amongst others.

In a particularly preferred embodiment of the present invention, the target gene is the BEV RNA-dependent RNA polymerase gene or a homologue, analogue or derivative thereof.

The animal cell in which expression of the target gene is modified may be any cell which is derived from a multicellular animal, including cell and tissue cultures thereof. Preferably, the animal cell is derived from an insect, reptile, amphibian, bird, human or other mammal. Exemplary animal cells include embryonic stem cells, cultured skin fibroblasts, neuronal cells, somatic cells, haematopoietic stem cells, T-cells and immortalised cell lines such as COS, VERO, HeLa, mouse C127, Chinese hamster ovary (CHO), WI-38, baby hamster kidney (BHK) or MDBK cell lines, amongst others. Such cells and cell lines are readily available to those skilled in the art. Accordingly, the tissue or organ in which expression of the target gene is modified may be any tissue or organ comprising such animal cells.

As used herein, the term "dispersed nucleic acid molecule" shall be taken to refer to a nucleic acid molecule which comprises a nucleotide sequence which is substantially identical or complementary to the nucleotide sequence of a gene which originates from the cell, tissue or organ into which said nucleic acid molecule is introduced, wherein said nucleic acid molecule is non-endogenous in the sense that it is introduced to the cell, tissue or organ of an animal via recombinant means and will generally be present as extrachromosomal nucleic acid or alternatively, as integrated chromosomal nucleic acid which is genetically-unlinked to said gene. More particularly, the "dispersed nucleic acid molecule" will comprise chromosomal or extrachromosomal nucleic acid which is unlinked to the target gene against which it is directed in a physical map, by virtue of their not being tandemly-linked or alternatively, occupying a different chromosomal position on the same chromosome or being localised on a different chromosome or present in the cell as an episome, plasmid, cosmid or virus particle.

By "foreign nucleic acid molecule" is meant an isolated nucleic acid molecule which has a nucleotide sequence which originates from the genetic sequence of an organism which is different from the organism to which the foreign nucleic acid molecule is introduced. This definition encompasses a nucleic acid molecule which originates from a different individual of the same lowest taxonomic grouping (i.e. the same population) as the taxonomic grouping to which said nucleic acid molecule is introduced, as well as a nucleic acid molecule which originates from a different individual of a different taxonomic grouping as the taxonomic grouping to which said nucleic acid molecule is introduced.

Accordingly, a target gene which comprises a foreign nucleic acid molecule may be a nucleic acid molecule which has been introduced from one organism to another organism using transformation or introgression technologies. Exemplary foreign nucleic acid molecules according to this embodiment of the invention include the green fluorescent protein-encoding gene derived from the jellyfish *Aequoria victoria* (Prasher *et al.*, 1992; International Patent Publication No. WO 95/07463), tyrosinase genes and in particular the murine tyrosinase gene (Kwon *et al.*, 1988), the *Escherichia coli lac*I gene which is capable of encoding a polypeptide

repressor of the *lac*Z gene or a homologue, analogue or derivative of said genes or a complementary nucleotide sequence thereto.

The present invention is further useful for simultaneously targeting the expression of several target genes which are co-expressed in a particular cell, for example by using a dispersed nucleic acid molecule or foreign nucleic acid molecule which comprises nucleotide sequences which are substantially identical to each of said co-expressed target genes.

By "substantially identical" is meant that the introduced dispersed or foreign nucleic acid molecule of the invention and the target gene sequence are sufficiently identical at the nucleotide sequence level to permit base-pairing there between under standard intracellular conditions.

Preferably, the dispersed or foreign nucleic acid molecule of the invention and the target gene sequence are at least about 80-85% identical at the nucleotide sequence level, more preferably at least about 85-90% identical, even more preferably at least about 90-95% identical and still even more preferably at least about 95-99% or 100% identical at the nucleotide sequence level to the target gene.

Preferably, the dispersed or foreign nucleic acid molecule which is introduced to the cell, tissue or organ comprises RNA or DNA.

More preferably, the dispersed or foreign nucleic acid molecule further comprises a nucleotide sequence or is complementary to a nucleotide sequence which is capable of encoding an amino acid sequence encoded by the target gene. Even more preferably, the nucleic acid molecule includes one or more ATG or AUG translational start codons.

In a particularly preferred embodiment of the invention, the dispersed or foreign nucleic acid molecule comprises a nucleotide sequence which is derived from the open reading frame of a viral polymerase gene, such as a retrovirus, lentivirus, Sinbis alphavirus or bovine

enterovirus (BEV) RNA-dependent RNA polymerase, HSV I DNA polymerase or bovine herpes virus DNA polymerase, amongst others or alternatively, a viral coat protein gene, such as the Sinbis alphavirus, bovine enterovirus, HSV I or bovine herpes virus coat protein genes.

In an alternative embodiment, the foreign or dispersed nucleic acid molecule comprises a nucleotide sequence which is derived from the murine tyrosinase gene.

In a further alternative embodiment, the foreign or dispersed nucleic acid molecule comprises a nucleotide sequence which is derived from the *E. coli lac*I gene.

In a further alternative embodiment, the foreign or dispersed nucleic acid molecule comprises a nucleotide sequence which is derived from the *A. victoria* GFP gene.

In still a further alternative embodiment of the invention, the foreign or dispersed nucleic acid molecule comprises one or more nucleotide sequences which are derived from the bovine enterovirus (BEV) RNA-dependent RNA polymerase gene and a nucleotide sequence which is derived from the *A. victoria* GFP gene in a single genetic construct and capable of being transcribed into a single mRNA molecule.

In yet still a further alternative embodiment of the invention, the foreign or dispersed nucleic acid molecule comprises one or more nucleotide sequences which are derived from the murine tyrosinase gene and the *E. coli lac*I gene in a single genetic construct and capable of being transcribed into a single mRNA molecule. Optionally, the foreign or dispersed nucleic acid molecule further comprises a nucleotide sequence which is derived from the *A. victoria* GFP gene.

The present invention is not in any way limited by the precise nucleotide sequence of the foreign or dispersed nucleic acid molecule or by the number or configuration of target gene sequences therein which is/are used to modulate expression of the target gene and all such

sequences are referred to herein merely for the purposes of exemplification to demonstrate the efficacy of the inventive method.

Standard methods may be used to introduce the dispersed nucleic acid molecule or foreign nucleic acid molecule into the cell, tissue or organ for the purposes of modulating the expression of the target gene. For example, the nucleic acid molecule may be introduced as naked DNA or RNA, optionally encapsulated in a liposome, in a virus particle as attenuated virus or associated with a virus coat or a transport protein or inert carrier such as gold or as a recombinant viral vector or bacterial vector or as a genetic construct, amongst others.

Administration means include injection and oral ingestion (e.g. in medicated food material), amongst others.

The subject nucleic acid molecules may also be delivered by a live delivery system such as using a bacterial expression system optimised for their expression in bacteria which can be incorporated into gut flora. Alternatively, a viral expression system can be employed. In this regard, one form of viral expression is the administration of a live vector generally by spray, feed or water where an infecting effective amount of the live vector (e.g. virus or bacterium) is provided to the animal. Another form of viral expression system is a non-replicating virus vector which is capable of infecting a cell but not replicating therein. The non-replicating viral vector provides a means of introducing to the human or animal subject genetic material for transient expression therein. The mode of administering such a vector is the same as a live viral vector.

The carriers, excipients and/or diluents utilised in delivering the subject nucleic acid molecules to a host cell should be acceptable for human or veterinary applications. Such carriers, excipients and/or diluents are well-known to those skilled in the art. Carriers and/or diluents suitable for veterinary use include any and all solvents, dispersion media, aqueous solutions, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. Except insofar as any conventional media or agent is incompatible with the active

ingredient, use thereof in the composition is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

In an alternative embodiment, the invention provides a method of modulating the expression of a target gene in an animal cell, tissue or organ, said method at least comprising the steps of:

(i) selecting one or more dispersed nucleic acid molecules or foreign nucleic acid molecules which comprise a nucleotide sequence which is substantially identical to the nucleotide sequence of said target gene or a region thereof or which is complementary thereto; and

(ii) introducing said dispersed nucleic acid molecules or foreign nucleic acid molecules to said cell, tissue or organ for a time and under conditions sufficient for translation of the mRNA product of said target gene to be modified, subject to the proviso that the transcription of said mRNA product is not exclusively repressed or reduced.

To select appropriate nucleotide sequences for targeting expression of the target gene, several approaches may be employed. In one embodiment, specific regions of characterised genes may be cloned in operable connection with a suitable promoter and assayed for efficacy in reducing target gene expression in animal cells. Alternatively, shotgun libraries of genetic sequences may be produced and assayed for their efficacy in reducing target gene expression. The advantage associated with the latter approach is that it is not necessary to have any prior knowledge of the significance of any particular target gene in specifying an undesirable phenotype in the animal cell. For example, shotgun libraries comprising virus sub-genomic fragments may be employed and tested directly for their ability to confer virus immunity on the animal host cell, without prior knowledge of the role which any virus genes play in pathogenesis of the host cell.

As used herein, the term "shotgun library" is a set of diverse nucleotide sequences wherein each member of said set is preferably contained within a suitable plasmid, cosmid,

bacteriophage or virus vector molecule which is suitable for maintenance and/or replication in a cellular host. The term "shotgun library" includes a representative library, in which the extent of diversity between the nucleotide sequences is numerous such that all sequences in the genome of the organism from which said nucleotide sequences is derived are present in the "set" or alternatively, a limited library in which there is a lesser degree of diversity between said sequences. The term "shotgun library" further encompasses random nucleotide sequences, wherein the nucleotide sequence comprises viral or cellular genome fragments, amongst others obtained for example by shearing or partial digestion of genomic DNA using restriction endonucleases, amongst other approaches. A "shotgun library" further includes cells, virus particles and bacteriophage particles comprising the individual nucleotide sequences of the diverse set.

Preferred shotgun libraries according to this embodiment of the invention are "representative libraries", comprising a set of nucleotide sequences derived from a viral pathogen of an animal or alternatively, derived from an animal cell.

In a particularly preferred embodiment of the invention, the shotgun library comprises cells, virus particles or bacteriophage particles comprising a diverse set of nucleotide sequences which encode a diverse set of amino acid sequences, wherein the member of said diverse set of nucleotide sequences are placed operably under the control of a promoter sequence which is capable of directing the expression of said nucleotide sequence in an animal cell.

Accordingly, the nucleotide sequence may comprise at least about 1 to 200 nucleotides in length. The use of larger fragments, particularly employing randomly sheared nucleic acid derived from viral or animal genomes, is not excluded.

The introduced nucleic acid molecule is preferably in an expressible form.

By "expressible form" is meant that the subject nucleic acid molecule is presented in an arrangement such that it may be expressed in an animal cell, tissue, organ or whole organism,

at least at the transcriptional level (i.e. it is expressed in the animal cell to yield at least an mRNA product which is optionally translatable or translated to produce a recombinant peptide, oligopeptide or polypeptide molecule).

By way of exemplification, in order to obtain expression of the dispersed nucleic acid molecule or foreign nucleic acid molecule in the cell, tissue or organ of interest a synthetic gene or a genetic construct comprising said synthetic gene is produced, wherein said synthetic gene comprises a nucleotide sequence as described *supra* in operable connection with a promoter sequence which is capable of regulating expression therein. Thus, the subject nucleic acid molecule will be operably connected to one or more regulatory elements sufficient for eukaryotic transcription to occur.

Accordingly, a further alternative embodiment of the invention provides a method of modulating the expression of a target gene in an animal cell, tissue or organ, said method at least comprising the steps of:

(i) selecting one or more dispersed nucleic acid molecules or foreign nucleic acid molecules which comprise a nucleotide sequence which is substantially identical to the nucleotide sequence of said target gene or a region thereof or which is complementary thereto;

(ii) producing a synthetic gene comprising said dispersed nucleic acid molecules or foreign nucleic acid molecules or a genetic construct comprising same;

(iii) introducing said synthetic gene or genetic construct to said cell, tissue or organ; and

(iv) expressing said synthetic gene or genetic construct in said cell, tissue or organ for a time and under conditions sufficient for translation of the mRNA product of said target gene to be modified, subject to the proviso that the transcription of said mRNA product is not exclusively repressed or reduced.

Hereinafter, the term "structural gene region" shall be taken to refer to that part of a synthetic gene which comprises a dispersed nucleic acid molecule or foreign nucleic acid molecule as

described herein which is expressed in a cell, tissue or organ under the control of a promoter sequence to which it is operably connected. A structural gene region may comprise one or more dispersed nucleic acid molecules and/or foreign nucleic acid molecules operably under the control of a single promoter sequence or multiple promoter sequences. Accordingly, the structural gene region of a synthetic gene may comprise a nucleotide sequence which is capable of encoding an amino acid sequence or is complementary thereto. In this regard, a structural gene region which is used in the performance of the instant invention may also comprise a nucleotide sequence which encodes an animo acid sequence yet lacks a functional translation initiation codon and/or a functional translation stop codon and, as a consequence, does not comprise a complete open reading frame. In the present context, the term "structural gene region" also extends to a non-coding nucleotide sequences, such as 5′- upstream or 3′- downstream sequences of a gene which would not normally be translated in a eukaryotic cell which expresses said gene.

Wherein the structural gene region comprises more than one dispersed nucleic acid molecule or foreign nucleic acid molecule it shall be referred to herein as a "multiple structural gene region" or similar term. The present invention clearly extends to the use of multiple structural gene regions which preferably comprise a direct repeat sequence, inverted repeat sequence or interrupted palindrome sequence of a particular structural gene or a fragment thereof.

Reference herein to a "gene" or "genes" is to be taken in its broadest context and includes:

(i) a classical genomic gene consisting of transcriptional and/or translational regulatory sequences and/or a coding region and/or non-translated sequences (i.e. introns, 5′- and 3′- untranslated sequences); and/or

(ii) mRNA or cDNA corresponding to the coding regions (i.e. exons) and 5′- and 3′- untranslated sequences of the gene; and/or

(iii) a structural region corresponding to the coding regions (i.e. exons) optionally further comprising untranslated sequences and/or a heterologous promoter sequence which consists of transcriptional and/or translational regulatory regions capable of conferring expression

characteristics on said structural region.

The term "gene" is also used to describe synthetic or fusion molecules encoding all or part of a functional product.

Accordingly, in the context of the present invention, a structural gene region may also comprise a fusion between two or more open reading frames of the same or different genes. In such embodiments, the invention may be used to modulate the expression of one gene, by targeting different non-contiguous regions thereof or alternatively, to simultaneously modulate the expression of several different genes, including different genes of a multigene family. In the case of a fusion nucleic acid molecule which is non-endogenous to the animal cell and in particular comprises two or more nucleotide sequences derived from a viral pathogen, the fusion may provide the added advantage of conferring simultaneous immunity or protection against several pathogens, by targeting the expression of genes in said several pathogens. Alternatively or in addition, the fusion may provide more effective immunity against any pathogen by targeting the expression of more than one gene of that pathogen.

Particularly preferred structural gene regions according to this aspect of the invention are those which include at least one translatable open reading frame, more preferably further including a translational start codon located at the 5'-end of said open reading frame, albeit not necessarily at the 5'-terminus of said structural gene region. In this regard, notwithstanding that the structural gene region may comprise at least one translatable open reading frame and/or AUG or ATG translational start codon, the inclusion of such sequences in no way suggests that the present invention requires translation of the introduced nucleic acid molecule to occur in order to modulate the expression of the target gene. Whilst not being bound by any theory or mode of action, the inclusion of at least one translatable open reading frame and/or translational start codon in the subject nucleic acid molecule may serve to increase stability of the mRNA transcription product thereof, thereby improving the efficiency of the invention.

Wherein the synthetic gene or genetic construct is expressed, it is particularly preferred that the structural gene region or multiple structural gene region thereof comprising the dispersed or foreign nucleic acid molecule is expressed prior to expression of the target gene or alternatively, simultaneous with transcription of the mRNA product of the target gene.

Depending on the duration and relative levels of expression of the synthetic gene and target gene, an animal cell carrying both sequences will exhibit a variety of different phonotypic traits. In particular, selecting cells, tissues or organs which express varying phenotypes can be readily achieved in accordance with the present invention.

In consideration of the preferred requirement for high-level expression which coincides with expression of the target gene or precedes expression of the target gene, it is highly desirable that the promoter sequence is a constitutive strong promoter such as the CMV-IE promoter or the SV40 early promoter sequence or SV40 late promoter sequence, amongst others.

For expression in eukaryotic cells, the synthetic gene or genetic construct generally comprises, in addition to the nucleic acid molecule of the invention, a promoter and optionally other regulatory sequences designed to facilitate expression of the dispersed nucleic acid molecule or foreign nucleic acid molecule.

Reference herein to a "promoter" is to be taken in its broadest context and includes the transcriptional regulatory sequences of a classical genomic gene, including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or in a tissue-specific manner. A promoter is usually, but not necessarily, positioned upstream or 5′, of a structural gene region, the expression of which it regulates. Furthermore, the regulatory elements comprising a promoter are usually positioned within 2 kb of the start site of transcription of the gene.

In the present context, the term "promoter" is also used to describe a synthetic or fusion molecule, or derivative which confers, activates or enhances expression of a nucleic acid molecule in a cell.

Preferred promoters may contain additional copies of one or more specific regulatory elements, to further enhance expression of the sense molecule and/or to alter the spatial expression and/or temporal expression of said sense molecule. For example, regulatory elements which confer copper inducibility may be placed adjacent to a heterologous promoter sequence driving expression of a sense molecule, thereby conferring copper inducibility on the expression of said molecule.

Placing a nucleic acid molecule under the regulatory control of a promoter sequence means positioning the said molecule such that expression is controlled by the promoter sequence. Promoters are generally positioned 5′ (upstream) to the genes that they control. In the construction of heterologous promoter/structural gene combinations it is generally preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting, i.e., the gene from which the promoter is derived. As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the preferred positioning of a regulatory sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting, i.e., the genes from which it is derived. Again, as is known in the art, some variation in this distance can also occur.

Examples of promoters suitable for use in producing synthetic genes or genetic constructs include viral, fungal, bacterial, animal and plant derived promoters capable of functioning in animal cell, in particular a human, insect, fish, crustacean, mollusc, avian, reptile or mammalian cell. The promoter may regulate the expression of the said molecule constitutively, or differentially with respect to the tissue in which expression occurs or, with respect to the developmental stage at which expression occurs, or in response to external

stimuli such as physiological stresses, or plant pathogens, or metal ions, amongst others.

Preferably, the promoter is capable of regulating expression of a structural gene region or multiple structural gene region in a cell derived from an mammal or a human.

In a particularly preferred embodiment, the promoter is the CMV-IE or the SV40 early promoter sequence or SV40 late promoter sequence or the RSV1 LTR promoter or a promoter sequence derived therefrom.

In a preferred embodiment of the invention, a structural gene region or multiple structural gene region is placed in the "sense" orientation relative to the promoter sequence, such that when it is transcribed an mRNA product is synthesized which, if translated, is capable of encoding a polypeptide product of the target gene or a fragment thereof.

However, the present invention is not to be limited to the use of such an arrangement and the invention clearly extends to the use of synthetic genes and genetic constructs wherein the a structural gene region or multiple structural gene region is placed in the "antisense" orientation relative to the promoter sequence, such that at least a part of the mRNA transcription product thereof is complementary to the mRNA encoded by the target gene or a fragment thereof.

In an alternative embodiment of the invention, the structural gene region or multiple structural gene region is operably connected to both a first promoter sequence and a second promoter sequence, wherein said promoters are located at the distal and proximal ends thereof such that said a structural gene region or multiple structural gene region is placed in the "sense" orientation relative to the first promoter sequence and in the "antisense" orientation relative to the second promoter sequence. According to this embodiment, it is also preferred that the first and second promoters be different, to prevent competition there between for cellular transcription factors which bind thereto. The advantage of this arrangement is that the effects of transcription from the first and second promoters in reducing target gene expression in the

cell may be compared to determine the optimum orientation for each nucleotide sequence tested.

In a further alternative embodiment, a genetic construct is used which comprises two or more structural gene regions or multiple structural gene regions wherein each of said structural gene regions is placed operably under the control of its own promoter sequence. As with other embodiments described herein, the orientation of each structural gene region may be varied to maximise its modulatory effect on target gene expression.

The synthetic gene or genetic construct comprising same preferably contains additional regulatory elements for efficient transcription, for example a transcription termination sequence.

The term "terminator" refers to a DNA sequence at the end of a transcriptional unit which signals termination of transcription. Terminators are 3'-non-translated DNA sequences containing a polyadenylation signal, which facilitates the addition of polyadenylate sequences to the 3'-end of a primary transcript. Terminators active in plant cells are known and described in the literature. They may be isolated from bacteria, fungi, viruses, animals and/or plants or synthesized *de novo*.

As with promoter sequences, the terminator may be any terminator sequence which is operable in the cells, tissues or organs in which it is intended to be used.

Particularly preferred terminators for use in the genetic constructs include mammalian cell-expressible terminator sequences such as the SPA terminator sequence, CMV-IE gene terminator and SV40 large T antigen gene terminator amongst others.

Those skilled in the art will be aware of how to produce the synthetic genes and genetic constructs described herein and of the requirements for obtaining the expression thereof, when so desired, in a specific cell or cell-type under the conditions desired. In particular,

it will be known to those skilled in the art that the genetic manipulations required to perform the present invention may require the propagation of a genetic construct described herein or a derivative thereof in a prokaryotic cell such as an *E. coli* cell or an animal cell.

As will be known to those skilled in the art, genetic constructs may further comprise genetic sequences corresponding to a bacterial origin of replication and/or a selectable marker gene such as an antibiotic-resistance gene, suitable for the maintenance and replication of said genetic construct in a prokaryotic or eukaryotic organism. Such sequences are well-known in the art. Usually, an origin of replication or a selectable marker gene suitable for use in bacteria is physically-separated from those genetic sequences contained in the genetic construct which are intended to be expressed or transferred to a eukaryotic cell, or integrated into the genome of a eukaryotic cell.

Means for transfecting or transforming animal cells with the synthetic genes described herein or a genetic construct comprising same are well-known to those skilled in the art.

The present invention extends to all genetic constructs essentially as defined herein, which include further genetic sequences intended for the maintenance and/or replication of said genetic construct in prokaryotes or eukaryotes and/or the integration of said genetic construct or a part thereof into the genome of a eukaryotic cell or organism.

As with dispersed or foreign nucleic acid molecules, standard methods described *supra* may be used to introduce synthetic genes and genetic constructs into the cell, tissue or organ for the purposes of modulating the expression of the target gene. Particularly, preferred methods suited to the introduction of synthetic genes and genetic constructs comprising same to eukaryotic cells include liposome-mediated transfection or transformation, transformation of cells with attenuated virus particles or bacterial cells and standard procedures for the transformation of animal cells, tissues, organs or organisms.

Genetic constructs are particularly suitable for the transformation of a eukaryotic cell to

introduce novel genetic traits thereto, in addition to the provision of resistance characteristics to viral pathogens. Such additional novel traits may be introduced in a separate genetic construct or, alternatively on the same genetic construct which comprises the synthetic genes described herein. Those skilled in the art will recognise the significant advantages, in particular in terms of reduced genetic manipulations and tissue culture requirements and increased cost-effectiveness, of including genetic sequences which encode such additional traits and the synthetic genes described herein in a single genetic construct.

In a further embodiment of the present invention, the synthetic genes and genetic constructs described herein are adapted for integration into the genome of a cell in which it is expressed. Those skilled in the art will be aware that, in order to achieve integration of a genetic sequence or genetic construct into the genome of a host cell, certain additional genetic sequences may be required.

The present invention clearly extends to isolated cells carrying the genetic constructs of the invention and to regenerated tissues, organs and whole organisms derived therefrom.

A variety of traits are selectable with appropriate procedures and sufficient numbers of transformed cells. Such traits include, but are not limited to, visible traits, disease-resistance traits, and pathogen-resistance traits. The modulatory effect is applicable to a variety of genes expressed in animals including, for example, endogenous genes responsible for cellular metabolism or cellular transformation, including oncogenes, transcription factors and other genes which encode polypeptides involved in cellular metabolism.

For example, an alteration in the pigment production in mice is engineered by targeting the expression of the tyrosinase gene therein. This provides a novel phenotype of albinism in black mice. Additionally, by targeting genes required for virus replication in an animal cell, the genetic construct of the instant invention which comprises a virus replicase, polymerase, coat protein or uncoating gene may be introduced into a cell where it is expressed, to confer immunity against the virus upon the cell.

Among the easiest genes to target for the performance of the invention are the viral pathogen genes, which are utilised to confer resistance on the animal cell, because of the smaller genome size of such organisms compared to animals. In particular, the smaller genome size means that small sub-genomic libraries are required to be produced to qualify as "representative libraries".

The introduced sequence generally will be substantially homologous to the endogenous sequence intended to be modulated, such that the controlling elements recognize that the introduced sequence is present, the interaction results in the modulatory effect. This minimal homology will typically be greater than about 85%, but a higher homology might exert a more effective modulation of expression of the endogenous sequences. Substantially greater homology, or more than about 90% is preferred, though about 95% to absolute identity would be most preferred. Consequently, the effect should apply to any other proteins within a similar family of genes exhibiting homology or substantial homology.

The introduced sequence, needing less than absolute homology, also need not be full length, relative to either the primary transcription product or fully processed mRNA. A higher homology in a shorter than full length sequence compensates for a longer less homologous sequence. Furthermore, the introduced sequence need not have the same intron or exon pattern, and homology of non-coding segments will be equally effective. Normally, a sequence of greater than 20-100 nucleotides should be used, though a sequence of greater than about 200-300 nucleotides would be preferred, and a sequence of greater than 500-1000 nucleotides would be especially preferred depending on the size of the endogenous gene.

In order to observe many novel traits, in particular those which are tissue-specific or organ-specific or developmentally-regulated, regeneration of a transformed cell carrying the synthetic genes and genetic constructs described herein will be required. those skilled in the art will be aware that this means growing a whole organism from a transformed animal cell, a group of such cells, a tissue or organ. Standard methods for the regeneration of certain animals from isolated cells are known to those skilled in the art.

The present invention is further described with reference to the following non-limiting Examples.

EXAMPLE 1

Genetic constructs

A) Genetic constructs comprising a BEV polymerase structural gene operably connected to the CMV-IE promoter sequence.

Isolation of BEV RNA polymerase gene fragments

The BEV RNA-dependent RNA polymerase coding region was amplified as a 1,385 bp DNA fragment from a full-length cDNA clone encoding same, using the primers designated BEV-1 (SEQ ID NO:1) and BEV-2 (SEQ ID NO:2), under standard amplification conditions. The amplified DNA contained a 5′-*Bgl* II restriction enzyme site, derived from the BEV-1 primer sequence and a 3′*Bam*HI restriction enzyme site, derived from the BEV-2 primer sequence. Additionally, as the BEV-1 primer sequence contains a translation start signal 5′-ATG-3′ engineered at positions 15-17 of SEQ ID NO:1, the amplified BEV polymerase structural gene comprises the start site in-frame with BEV polymerase-encoding nucleotide sequences, Thus, the amplified BEV polymerase structural gene comprises the ATG start codon immediately upstream (ie. juxtaposed) to the BEV polymerase-encoding sequence. There is no translation stop codon in the amplified DNA.

Additionally, the complete BEV polymerase coding region was amplified from a full-length cDNA clone encoding same, using primers BEV-1 (SEQ ID NO:1) and BEV-3 (SEQ ID NO:3). Primer BEV-3 comprises a *Bam*HI restriction enzyme site at positions 5 to 10 inclusive of SEQ ID NO:3 and the complement of a translation stop signal at positions 11 to 13 of SEQ ID NO:3. As a consequence, an open reading frame comprising a translation start signal and translation stop signal, contained between the *Bgl* II and *Bam*HI restriction sites.

Additionally, a non-translatable BEV polymerase structural gene was amplified from a full-

length BEV polymerase cDNA clone using the amplification primers BEV-3 (SEQ ID NO:3) and BEV-4 (SEQ ID NO:4). Primer BEV-4 comprises a *Bgl*II cloning site at positions 5-10 of SEQ ID NO:4 and sequences downstream of this *Bgl*II site are homologous to nucleotide sequences of the BEV polymerase gene. There is no functional ATG start codon in the amplified DNA product of primers BEV-3 and BEV-4.

1. Plasmid pEGFP.BEV.1

Plasmid pEGFP.BEV.1 (Figure 1) is capable of expressing the BEV polymerase structural gene as a GFP fusion polypeptide under the control of the CMV-IE promoter sequence.

2. Plasmid pCMV.BEV.2

Plasmid pCMV.BEV.2 (Figure 2) is capable of expressing the entire BEV polymerase open reading frame under the control of CMV-IE promoter sequence.

3. Plasmid pCMV.VEB

Plasmid pCMV.VEB (Figure 3) expresses an antisense BEV polymerase mRNA under the control of the CMV-IE promoter sequence.

4. Plasmid pCMV.BEVnt

Plasmid pCMV.BEVnt (Figure 4) expresses a non-translatable BEV polymerase structural gene in the sense orientation under the control of the CMV-IE promoter sequence.

5. Plasmid pCMV.BEVx2

Plasmid pCMV.BEVx2 (Figure 5) comprises a direct repeat of a complete BEV polymerase open reading frame under the control of the CMV-IE promoter sequence. In eukaryotic cells at least, the open reading frame located nearer the CMV-IE promoter is translatable.

6. Plasmid pCMV.BEV.VEB

Plasmid pCMV.BEV.VEB (Figure 6) comprises an inverted repeat or palindrome of a complete BEV polymerase open reading frame under the control of the CMV-IE promoter

sequence. In eukaryotic cells at least, the open reading frame located nearer the CMV-IE promoter is translatable.

7. Plasmid pCMV.BEV.GFP.VEB

Plasmid pCMV.BEV.GFP.VEB (Figure 7) is similar to plasmid pCMV.BEV.VEB except that the BEV structural gene inverted repeat or palindrome is interrupted by the insertion of a GFP open reading frame (stuffer fragment) therein. The BEV polymerase structural gene nearer the CMV-IE promoter sequence in plasmid pCMV.BEV.GFP.VEB is capable of being translated, at least in eukaryotic cells.

B) Genetic constructs comprising BEV polymerase structural genes operably connected to multiple promoter sequences

1. Plasmid pCMV.BEV.SV40L-O

Plasmid pCMV.BEV.SV40L-O (Figure 8) comprises a translatable BEV polymerase structural gene inserted in the sense orientation between the CMV-IE promoter and the SV40 late promoter sequences.

2. Plasmid pCMV.O.SV40L.BEV

Plasmid pCMV.O.SV40L.BEV (Figure 9) comprises a translatable BEV polymerase structural gene cloned downstream of tandem CMV-IE promoter and SV40 late promoter sequences.

3. Plasmid pCMV.O.SV40L.VEB

Plasmid pCMV.O.SV40L.VEB (Figure 10) comprises an antisense BEV polymerase structural gene cloned downstream of tandem CMV-IE promoter and SV40 late promoter sequences.

4. Plasmid pCMV.BEV.SV40L.BEV

Plasmid pCMV.BEV.SV40L.BEV(Figure 11) comprises a multiple structural gene unit

comprising two BEV polymerase structural genes placed operably and separately under control of the CMV-IE promoter and SV40 late promoter sequences.

5. Plasmid pCMV.BEV.SV40L.VEB

Plasmid pCMV.BEV.SV40L.VEB (Figure 12) comprises a multiple structural gene unit comprising two BEV polymerase structural genes placed operably and separately under control of the CMV-IE promoter and SV40 late promoter sequences. In this plasmid, the BEV polymerase structural gene is expressed in the sense orientation under control of the CMV-IE promoter to produce a translatable mRNA, whilst the BEV polymerase structural gene is also expressed under control of the SV40 promoter to produce an antisense mRNA.

6. Plasmid pCMV.SV40LR.cass

Plasmid pCMV.SV40LR.cass (Figure 13) comprises the synthetic poly(A) site and the SV40 late promoter sequence, sub-cloned such that the CMV-IE and the SV40 late promoters drive transcription, in the opposite direction, of a structural gene or a multiple structural gene unit which is inserted into the multiple cloning site of this plasmid vector. A *Bgl*II site is positioned between the opposing CMV- IE and SV40 late promoter sequences in this plasmid.

7. Plasmid pCMV.BEV.SV40LR

Plasmid pCMV.BEV.SV40LR (Figure 14) comprises a structural gene comprising the entire BEV polymerase open reading frame placed operably and separately under control of opposing CMV-IE promoter and SV40 late promoter sequences, thereby potentially producing BEV polymerase transcripts at least from both strands of the full-length BEV polymerase structural gene. To produce plasmid pCMV.BEV.SV40LR, a translatable BEV polymerase structural gene into the unique *Bgl*II site of plasmid pCMV.SV40LR.cass (Figure 13), such that the BEV open reading frame is present in the sense orientation relative to the CMV-IE promoter sequence.

C) Genetic constructs comprising the tyrosinase gene or a fragment thereof

Isolation of the tyrosinase open reading frame

The tyrosinase structural gene is isolated by polymerase chain reaction, from mRNA derived from murine cells, using the following oligonucleotide primers under standard polymerase chain reaction conditions:

Tyr 5'(forward primer; SEQ ID NO:5):

5'-CCCGGGGCTTAGTGTAAAACAGGCTGAGAG-3'; and

Tyr 3' (reverse primer; SEQ ID NO:6):

5'-CCCGGGCAAATCCCAGTCATTTCTTAGAAAC-3'.

Nucleotide residues 1 to 6 in each primer represent a *Sma*I cloning site. Nucleotides 7 to 30 of primer Tyr 5' correspond to the 5'-end of the murine tyrosinase cDNA sequence disclosed in GenBank Accession No. M20234 (Kwon *et al*, 1988). Nucleotides 7 to 31 of primer Tyr 3' correspond to the complement of the nucleotide sequence of the 3'-end of the murine tyrosinase cDNA sequence.

Isolation of the OPRSV1 promoter

A DNA fragment comprising the OPRSVI promoter, SV40 intron, *lac* operator sequence, multiple cloning site (MCS) and thymidine kinase (TK) poly(A) sequence was excised from plasmid pOPRSVI/MCS (Stratagene).

1. Plasmid pCMV.TYR

Plasmid pCMV.TYR (Figure 15) comprises the complete mouse tyrosinase cDNA sequence placed operably in connection, in the sense orientation, with the CMV-IE promoter sequence and upstream of the SV40 polyadenylation sequence.

2. Plasmid pCMV.TYRLIB

Plasmid pCMV.TYRLIB (Figure 16) comprises a structural gene or multiple structural gene unit

which comprises one or more tyrosinase gene fragments of approximately100 to 200 base pairs in length each, placed operably in connection with the CMV-IE promoter sequence and upstream of the SV40 polyadenylation signal. To produce pCMV.TYRLIB, blunt-ended fragments of the tyrosinase gene are produced, for example, by sonication or mechanical shearing and end-repair using T4 DNA polymerase and cloned in operable connection with the CMV IE promoter. Accordingly, the structural gene insert in plasmid pCMV.TYRLIB is variable and an representative library of pCMV.TYRLIB plasmids, covering the complete tyrosinase gene sequence, may be produced using such procedures. The present invention clearly encompasses such representative libraries.

Those skilled in the art will recognise that such procedures are also useful for structural genes other than tyrosinase and, as a consequence, the present invention clearly extends to synthetic genes and genetic constructs wherein the structural gene present in pCMV.TYRLIB is a structural gene other than a tyrosinase gene fragment.

3. Plasmid pCMVLacI.OPRSV1.GFP.TYR

Plasmid pCMVLacI.OPRSV1.GFP.TYR (Figure 17) is a dual construct in which the CMV IE promoter drives expression of the *lac*I gene and the mRNA of the mouse tyrosinase cDNA or a fragment thereof, whilst the OPRSVI promoter drives expression of GFP operably under control of the *lac*I gene. The construct is designed such that the mouse tyrosinase gene is fused to the 3 ' untranslated region of the *lac*I gene via a unique *Bsa*B1 cloning site. This cloning site is located after the stop codon of the *lac*I coding sequence, but before the SV40 polyadenylation signal. The construct also contains the hygromycin-resistance gene as a selection marker.

D) Genetic constructs comprising the *lac*I and green fluorescent protein (GFP) open reading frames

Isolation of the GFP coding region

The GFP open reading frame was amplified from pEGFP-N1 MCS (Clontech) as a *Xho*I-to-*Not*I fragment.

Isolation of the *lac*I coding region

The *Lac*I coding region was isolated from plasmid pCMV.LacI (Stratagene) as a *Hin*dIII- to-*Bsa*B1 fragment.

1. Plasmid pCMVLacI.OPRSV1.GFP.cass

Plasmid pCMVLacI.OPRSVI.GFP.cass (Figure 18) comprises the GFP coding region cloned immediately downstream of the OPRSV1 promoter and the *lac*I structural gene operably under control of the CMV IE promoter sequence. This plasmid is designed such that a structural gene or multiple structural gene unit can be fused to the 3 '-untranslated region of the *lac*I gene, by cloning directly into the unique *Bsa*B1 cloning site which is located after the *lac*I stop codon and before an SV40 polyadenylation signal, thereby placing expression of said structural gene or multiple structural gene operably under control of the CMV IE promoter, such that it is co-expressed with the *lac*I gene.

Alternatively, the *Bsa*B1 site may be modified to facilitate cloning of the structural gene or multiple structural gene unit downstream of the *lac*I gene, for example by the addition of linkers or adaptors.

This construct also contains the antibiotic selectable marker for hygromycin resistance.

D) Genetic constructs comprising the *lac*I open reading frame

1. Plasmid pCMV.Lac

Plasmid pCMV.Lac (Figure 19) contains a CMV IE promoter driving expression of the lac repressor protein encoded by the *Escherichia coli lac*I gene. Accordingly, the open reading frame of the *Lac*I gene is cloned in the sense orientation with respect to the CMV IE promoter sequence in this plasmid. This construct also contains the selectable marker for neomycin resistance.

EXAMPLE 2

Inactivation of virus gene expression in mammals.

Viral immune lines are created by expressing viral sequences in stably transformed cell lines.

In particular, lytic viruses are used for this approach since cell lysis provides very simple screens and also offer the ability to directly select for potentially rare transformation events which might create viral immunity. Sub-genomic fragments derived from a simple single stranded RNA virus (Bovine enterovirus - BEV) or a complex double stranded DNA virus, Herpes Simplex Virus I (HSV I) are cloned into a suitable vector and expressed in transformed cells. Mammalian cell lines are transformed with genetic constructs designed to express viral sequences driven by the strong cytomegalovirus (CMV-IE) promoter. Sequences utilised include specific viral replicase genes. Random "shotgun" libraries comprising repesentative viral gene sequences, may also be used and the introduced disspersed nucleic acid molcule, to target the expression of virus sequences.

Exemplary genetic constructs for use in this procedure, comprising nucleotide sequecnes derived from the BEV RNA-dependent RNA polymerase gene, are presented in Figures 1 to 14.

For viral polymerase constructs, large numbers (approximately 100) of transformed cell lines are generated and infected with the respective virus. For cells transformed with shotgun libraries very large numbers (hundreds) of transformed lines are generated and screened in bulk for viral immunity. Following virus challenge, resistant cell lines are selected and analysed further to determine the sequences conferring immunity thereon.

Resistant cell lines are supportive of the ability of the introduced nucleotide sequences to inactivate viral gene expression in a mammalian system.

Additionally, resistant lines obtained from such experiments are used to more precisely define molecular and biochemical characteristics of the modulation which is observed.

EXAMPLE 3

Inactivation of pigment biosynthesis in transgenic mice.

To investigate repression of gene expression in transgenic animals using the presently described invention, genetic constructs are produced to target expression of one or more pigment biosynthetic gene in transgenic mice. Pigment production in mice is well characterised genetically (Jackson, 1995). By targeting expression of the tyrosinase gene in transgenic mice, pigment production is inhibited. A simple visual assay for the efficacy of the invention measures the occurrence of albinism in genetically-black mice. Furthermore, since melanocytes can be readily cultured from mature animals this system offers the ability to undertake molecular analysis of gene inactivation events.

Constructs comprising a promoter such as the CMV promoter driving tyrosinase cDNA constructs are prepared and used to micro-inject mouse embryos.

Random shotgun libraries comprising representative nucleotide sequences of up to approximately 300bp in length each, collectively covering the entire tyrosinase gene sequence, may also be used to target the expression of the tyrosinase gene. This approach allows for selection of those nucleotide sequences which provide for optimum inactivation of tyrosinase gene expression. The random shotgun library may also be micro-injected into mouse embryos.

Exemplary genetic constructs for use in this procedure, targeting expression of the murine tyrosinase gene, are presented in Figures 15 to 17.

Gene inactivation events are monitored visually. Sectored gene inactivation events are easily detected by the occurrence of mottled or patchy colouration in transgenic animals. Animals showing evidence of extreme albinism or strong sectored albinism are selected. Melanocytes are cultured from such lines and subjected to more detailed analyses.

EXAMPLE 4

Inactivation of expression of nuclear genes using a simple visual reporter system.

To create a simple visual reporter system, cell lines are first stably transformed with marker constructs consisting of two genes, wherein the first of said genes encodes a trans-regulatory protein, for example the lac repressor which is normally capable of repressing the expression of the *Escherichia coli lacZ* gene and wherein the second gene corresponds to a visual marker gene, for example the green fluorescent protein (GFP) or tyrosinase gene placed operably in connection with a promoter comprising operator sequences for binding of said repressor.

To detect repression of expression of the reporter gene, the expression of the repressor gene is targeted by introducing a further genetic construct comprising a third nucleic acid molecule which is capable of repressing, reducing or repressing expression of said first gene. As a consequence, reporter gene expression is induced in cells which do not express the trans-regulatory protein, permitting visual assay of the efficacy of the introduced nucleic acid molecule.

Cell lines are transformed with the marker construct. Cloned lines are selected which show little or no background expression of the reporter gene, but high levels of expression when induced by IPTG (for lac). Once characterised lines are established these are super-transformed with constructs expressing the third nucleic acid molecule which targets expression of the trans-regulatory repressor protein. Repression of expression of the first nucleic acid molecule is then monitored visually by measuring reporter gene expression levels. Cell lines in which such repression of gene expression is identified are purified for detailed analysis.

Cell lines with such easily scored markers also provide ideal systems for examining the effects of transient delivery of constructs as either gene cassettes, by using viral delivery systems or by direct delivery of one or more oligonucleotides or oligoribonucleotides.

EXAMPLE 5

Transcription-mediated inactivation of *lac*I gene expression in mammalian cells

Background

In the presence of lactose as the sole carbon source, the *E. coli lac* operon expresses large quantities of the enzyme β-galactosidase, which is encoded by the *lac*Z. The three polycistronic genes of the *lac* operon are under transcriptional control of the *lac* promoter-operator sequence, which in turn is under the negative control of the *lac* repressor; a DNA binding protein product of the *lac*I gene. In the presence of one of a variety of beta-galactosides, the affinity of the repressor protein for the lac promoter-operator sequence is lowered, allowing RNA polymerase to bind to the promoter region of the genes resulting in transcription of the *lac* operon.

To produce transcription-mediated inactivation of *lac*I gene expression in mammalian cells, the present inventors have utilised the transcriptional repression function of the *lac*I repressor gene as the target gene and a visual reporter gene to detect successful inactivation of gene expression.

Method

Two genetic constructs are produced as follows:

1. A first genetic construct is produced which contains two promoters, wherein the first promoter, for example CMV IE, drives expression of the *lac*I gene and the second promoter comprising *lac* operator sequences, for example the OPRSV1 promoter (a modified RSV-LTR promoter derived from the vector pOPRSV1/MCS [Stratagene]) drives expression of a visual reporter gene such as the GFP reporter gene. In such a system, when the *lac* repressor protein binds to the *lac* operator sequences, transcription of the visual reporter gene is inhibited. In the presence of IPTG, the *lac*I repressor no longer binds the *lac* operator sequence and the second promoter is capable of regulating transcription of the visual reporter gene. Accordingly, a positive phenotype (in particular visual reporter gene expression) may be monitored in transformed cells.

2. A second genetic construct is produced which comprises a third promoter sequence, for example the CMV IE promoter, driving expression of the *lac*I gene.

It is particularly preferred that the first and second genetic constructs contains different selectable marker genes to facilitate their co-selection in transformed cells, for example the genes encoding neomycin resistance and hygromycin resistance, amongst others.

In this system, the *lac*I gene of the first genetic construct comprises the target gene, whilst the *lac*I gene of the second genetic construct comprises the introduced dispersed or foreign nucleic acid molecule.

An exemplary genetic construct, suitable for use as the first genetic construct which is referred to herein, comprises the CMV IE promoter driving *lac*I gene expression and the OPRSV1 promoter driving expression of the GFP gene, as set forth in Figure 18. An exemplary genetic construct, suitable for use as the second genetic construct which is referred to herein, comprises the CMV IE promoter driving *lac*I gene expression, as set forth in Figure 19.

The first genetic construct is transformed into a suitable mammalian cell line, for example MDBK cells using standard techniques and transformed cell lines are selected which are able to derepress the *lac* operator, as determined by increased expression of the visual reporter gene when IPTG is added to the cell culture media.

Stable lines comprising the first genetic construct and which reproducibly derepress the *lac* operator in the presence of IPTG are 'supertransformed' with the second genetic construct. Cell lines which contain both genetic constructs are selected by their ability to grow on different selective media.

On the other hand, cell lines comprising only the second genetic construct are able to grow on only one selective medium. Such cells preferably produce translatable or non-translatable *lac*I mRNA constitutively, by virtue of the promoter used to drive *lac*I gene expression therein.

In the selected cell lines comprising both genetic constructs, inactivation of the *lac*I target gene of the first genetic construct and/or selective degradation of the *lac*I repressor mRNA by the presence or expression of the introduced dispersed or foreign nucleic acid molecule (i.e. second genetic construct) may be monitored by determining visual reporter gene expression levels (Table 1). In the absence or presence of IPTG, cells comprising both the first and second genetic construct fail to produce the *lac* repressor protein, the second promoter is derepressed constitutively and expression of the reporter gene does not require the presence of IPTG in the media.

TABLE 1

GENETIC CONSTRUCT INTRODUCED	RELATIVE REPORTER GENE EXPRESSION	
	No IPTG in media	Plus IPTG in media
Construct I only	-	+++
Construct II only	-	-
Constructs I and II	+++	+++

EXAMPLE 6

Transcription-mediated inactivation of endogenous gene expression in mammalian cells

To investigate transcriptional mediated inactivation of expression of cellular genes in a mammalian system, the present inventors have utilised the *lac*I repressor gene as an ancillary target gene and a visual reporter gene to detect successful inactivation of gene expression in Madin Darby Bovine Kidney (MDBK) cells. The primary target gene in these experiments is a cellular or endogenous gene, such as the murine tyrosinase gene.

Two genetic constructs are produced as follows:

1. A first genetic construct is produced which contains two promoters, wherein the

first promoter, for example CMV IE, drives expression of both the *lac*I gene and the fused endogenous gene of interest, and the second promoter comprising *lac* operator sequences, for example the OPRSV1 promoter (a modified RSV-LTR promoter derived from the vector pOPRSV1/MCS [Stratagene]) drives expression of a visual reporter gene such as the GFP reporter gene.

2. A second genetic construct is produced which comprises a third promoter sequence, for example the CMV IE promoter, driving expression of two fused structural genes, wherein the first structural gene comprises the *lac*I gene and the second structural gene comprises the endogenous gene of interest placed between said first structural gene and a transcription termination sequence.

It is particularly preferred that the first and second genetic constructs contains different selectable marker genes to facilitate their co-selection in transformed cells, for example the genes encoding neomycin resistance and hygromycin resistance, amongst others.

In this system, the *lac*I gene of the first genetic construct comprises the ancillary target gene and the endogenous gene of the first genetic construct comprises the primary target gene, whilst the *lac*I structural gene and endogenous structural gene of the second genetic construct comprise the introduced dispersed or foreign nucleic acid molecule.

An exemplary genetic construct, suitable for use as the first genetic construct which is referred to in this Example, comprises the CMV IE promoter driving *lac*I and murine tyrosinase gene expression and the OPRSV1 promoter driving expression of the GFP gene, as set forth in Figure 17. Exemplary genetic constructs, suitable for use as the second genetic construct which is referred to herein, comprise the CMV IE promoter driving *lac*I gene and murine tyrosinase gene expression. In this context, the murine tyrosinase-specific nucleic acid molecule may comprise the full gene sequence or a fragment thereof, such as a gene fragment which has been cloned into a random shotgun library of representative tyrosinase gene fragments.

In use, the first genetic construct is transformed into a suitable mammalian cell line, for example MDBK cells using standard techniques and transformed cell lines are selected which are able to derepress the *lac* operator, as determined by increased expression of the visual reporter gene when IPTG is added to the cell culture media.

Stable lines comprising the first genetic construct and which reproducibly derepress the *lac* operator in the presence of IPTG are 'supertransformed' with the second genetic construct. Cell lines which contain both genetic constructs are selected by their ability to grow on different selective media. On the other hand, cell lines comprising only the first or second genetic construct are able to grow on only one selective medium.

In cells transformed with the first genetic construct alone, mRNA encoding the *lac*I repressor protein and the endogenous gene of interest is produced as a single transcript, wherein at least the *lac*I mRNA is further capable of being translated. In such cells, when the *lac*I mRNA is translated to produce a functional *lac* repressor protein, the *lac* repressor protein binds to the *lac* operator sequences present in the second promoter, such that transcription of the visual reporter gene is inhibited. Accordingly, in such circumstances, transcription of the endogenous gene is linked to the absence of detectable reporter gene expression. In the presence of IPTG, the *lac*I repressor no longer binds the *lac* operator sequence and the second promoter is capable of regulating transcription of the visual reporter gene, however reporter gene expression may be independent of transcription of the endogenous gene. Accordingly, a positive phenotype (in particular visual reporter gene expression) may be monitored in transformed cells only in the absence of IPTG.

In cells transformed with the second genetic construct alone, there is no visual reporter gene and, as a consequence, expression of the visual reporter gene will not be detected. However, mRNA encoding the endogenous gene may be detected in such cells, irrespective of whether IPTG is added to the culture medium.

In cells transformed with both the first and second genetic constructs, expression of both the

visual reporter gene and the endogenous gene is independent of IPTG concentration in the culture medium, wherein expression of the visual reporter gene is detectable both in the presence and absence of IPTG and expression of the endogenous gene is undetectable both in the presence and absence of IPTG (Table 2), suggesting that inactivation of both the ancillary and primary target genes has occurred. Absent inactivation, it would be expected that a significant proportion of cells would express the endogenous gene at a detectable level and fail to express the visual reporter gene.

TABLE 2

GENETIC CONSTRUCT INTRODUCED	RELATIVE GENE EXPRESSION			
	VISUAL REPORTER GENE		ENDOGENOUS GENE	
	No IPTG	Plus IPTG	No IPTG	Plus IPTG
Construct I only	-	+++	+++	+++
Construct II only	-	-	+++	+++
Construct I and II	+++	+++	-	-

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: AgGene Australia Pty.Ltd and The Government of Queensland as represented by Queensland Department of Primary Industries

ii) TITLE OF INVENTION: Synthetic genes and genetic constructs comprising same I

iii) NUMBER OF SEQUENCES: 6

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: DAVIES COLLISON CAVE
(B) STREET: 1 LITTLE COLLINS STREET
(C) CITY: MELBOURNE
(D) STATE: VICTORIA
(E) COUNTRY: AUSTRALIA
(F) ZIP: 3000

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:AU provisional
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: HUGHES EL, JOHN E L

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: +61 3 9254 2777
(B) TELEFAX: +61 3 9254 2770
(C) TELEX: AA 31787

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CGGCAGATCT AACAATGGCA GGACAAATCG AGTACATC 38

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCCGGGATCC TCGAAAGAAT CGTACCACTT C 31

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGCGGATCC TTAGAAAGAA TCGTACCAC 29

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CGGCAGATCT GGACAAATCG AGTACATC 28

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCCGGGGCTT AGTGTAAAAC AGGCTGAGAG 30

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCCGGGCAAA TCCCAGTCAT TTCTTAGAAA C 31

EQUIVALENTS

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

REFERENCES

1. Dorer, D.R., and Henikoff, S. (1994) *Cell* **7**: 993-1002.

2. Jackson, I.J. (1995) *Ann. Rev. Genet.* **28**: 189-217.

3. Kwon, B.S. *et al.* (1988) *Biochem. Biophys. Res. Comm.* **153**:1301- 1309.

4. Pal-Bhadra, M. *et al.* (1997) *Cell* **90**: 479-490.

5. Prasher, D.C. *et al.* (1992) *Gene* **111**: 229-233.

DATED this 19TH day of MARCH, 1998

Ag-Gene Australia Ltd AND

State of Queensland through its Department of Primary Industries

by DAVIES COLLISON CAVE

Patent Attorneys for the Applicants

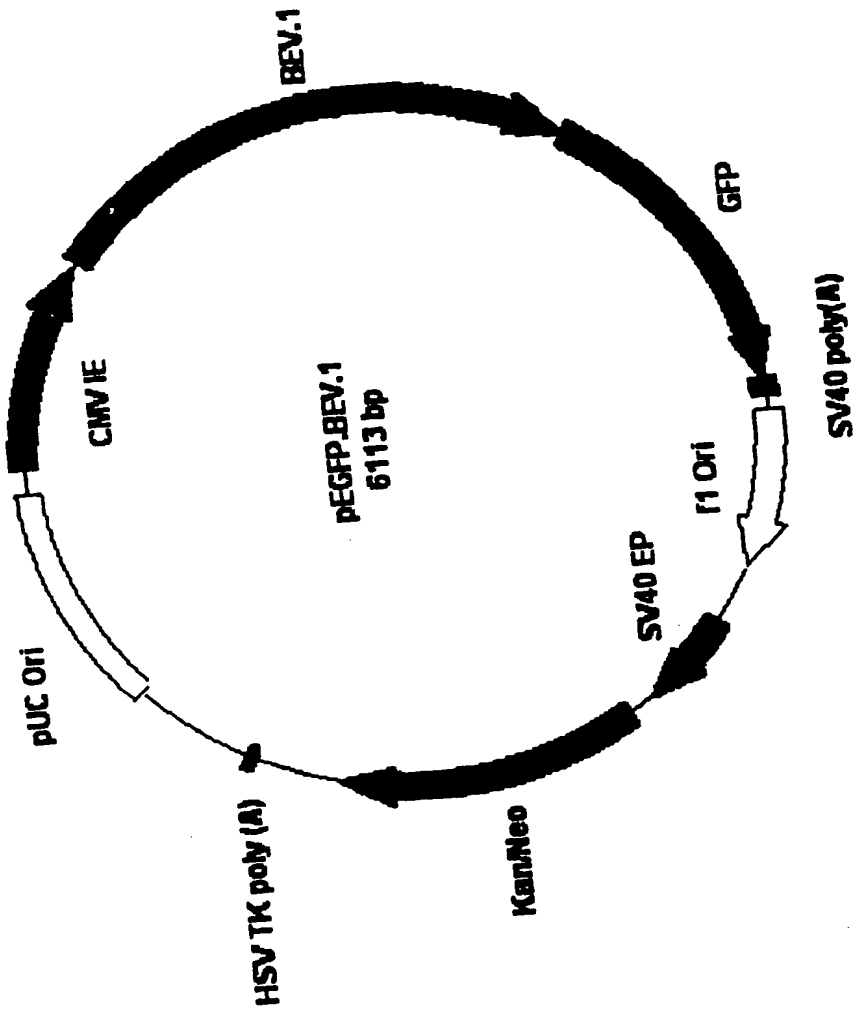
BEV.1
GFP
SV40 poly(A)
f1 Ori
SV40 EP
KanNeo
HSV TK poly (A)
pUC Ori
CMV IE
pEGFP-BEV.1
6113 bp

FIGURE 1

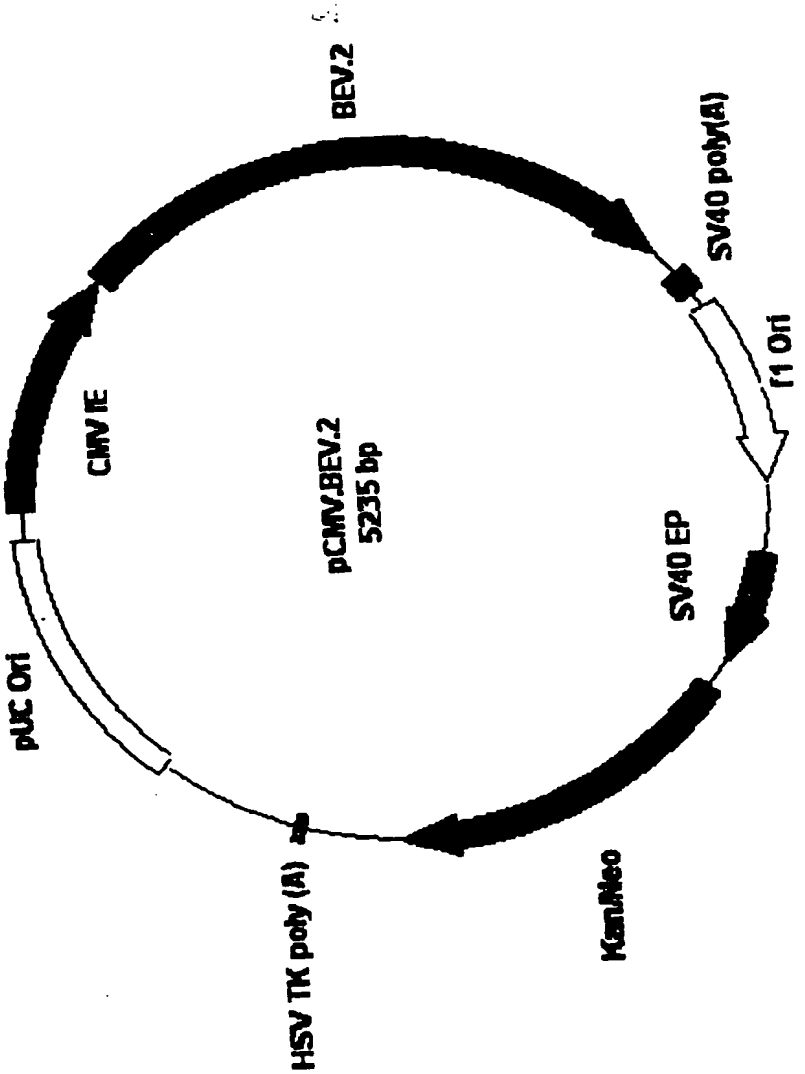
BEV.2
SV40 poly(A)
f1 Ori
CMV IE
pCMV.BEV.2
5235 bp
SV40 EP
pUC Ori
HSV TK poly (A)
KanNeo

FIGURE 2

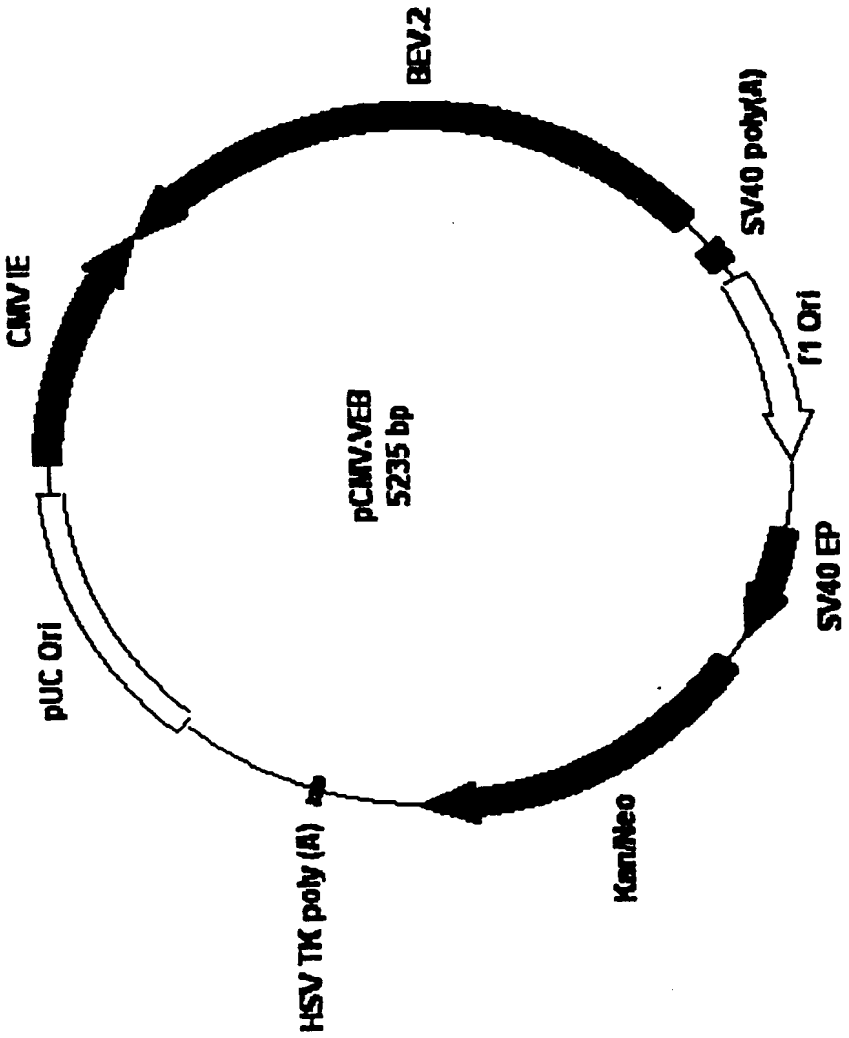
BEV2
SV40 poly(A)
f1 Ori
SV40 EP
KanNeo
HSV TK poly (A)
pUC Ori
CMV IE
pCMV.VEB
5235 bp

FIGURE 3

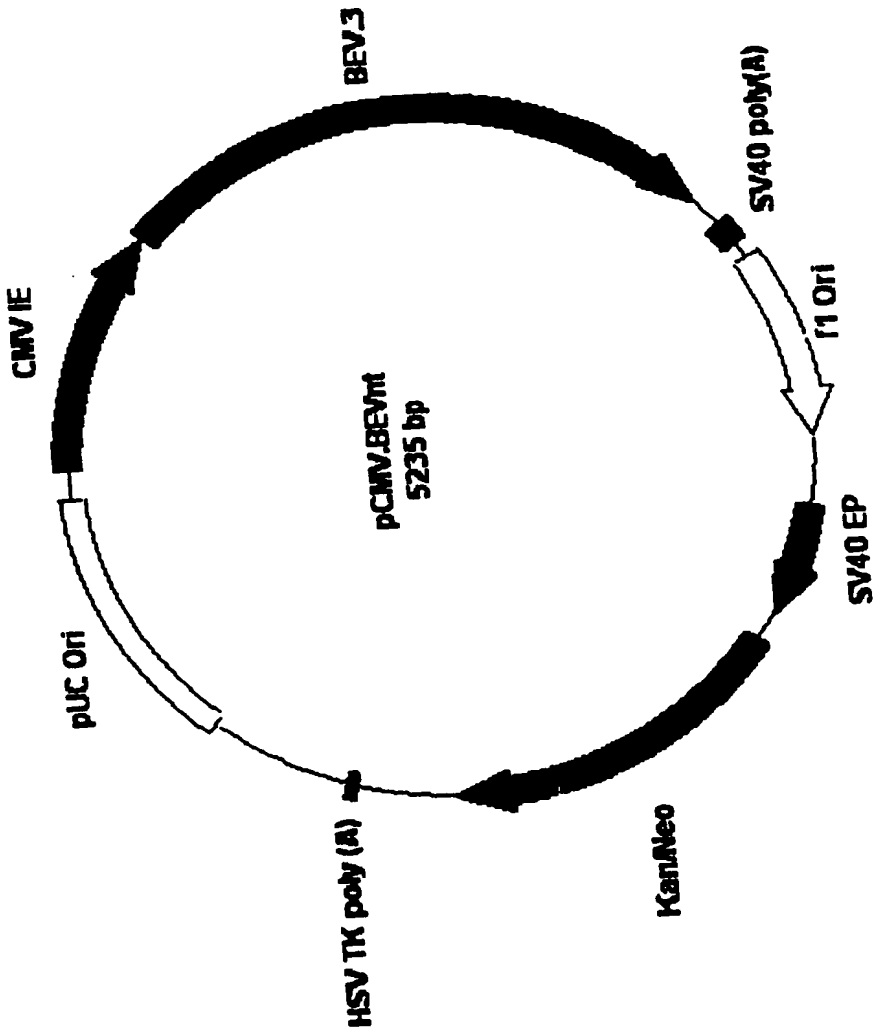
BEV3
SV40 poly(A)
f1 Ori
SV40 EP
KanNeo
HSV TK poly (A)
pUC Ori
CMV IE
pCMV.BEVmt
5235 bp

FIGURE 4

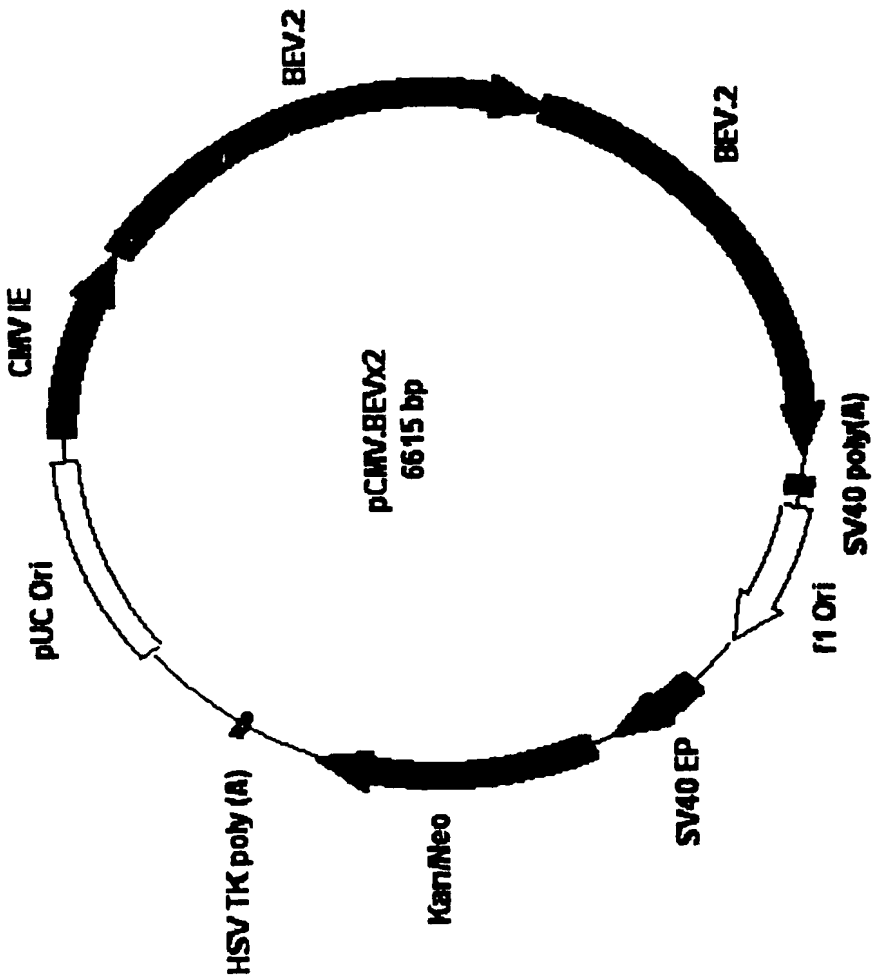
BEV.2
BEV.2
CMV IE
pCMV.BEVx2
6615 bp
SV40 poly(A)
pUC Ori
f1 Ori
HSV TK poly (A)
Kan/Neo
SV40 EP

FIGURE 5

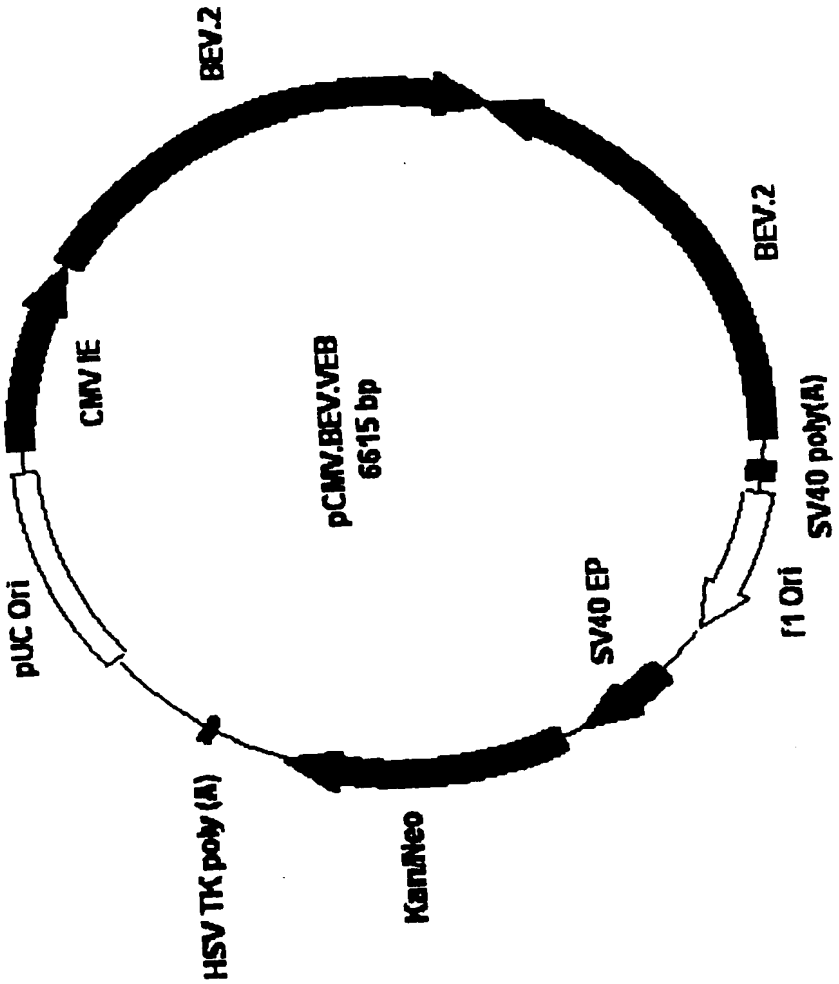
BEV.2
BEV.2
CMV IE
pCMV.BEV.VEB
6615 bp
SV40 poly(A)
pUC Ori
SV40 EP
f1 Ori
HSV TK poly (A)
KanNeo

FIGURE 6

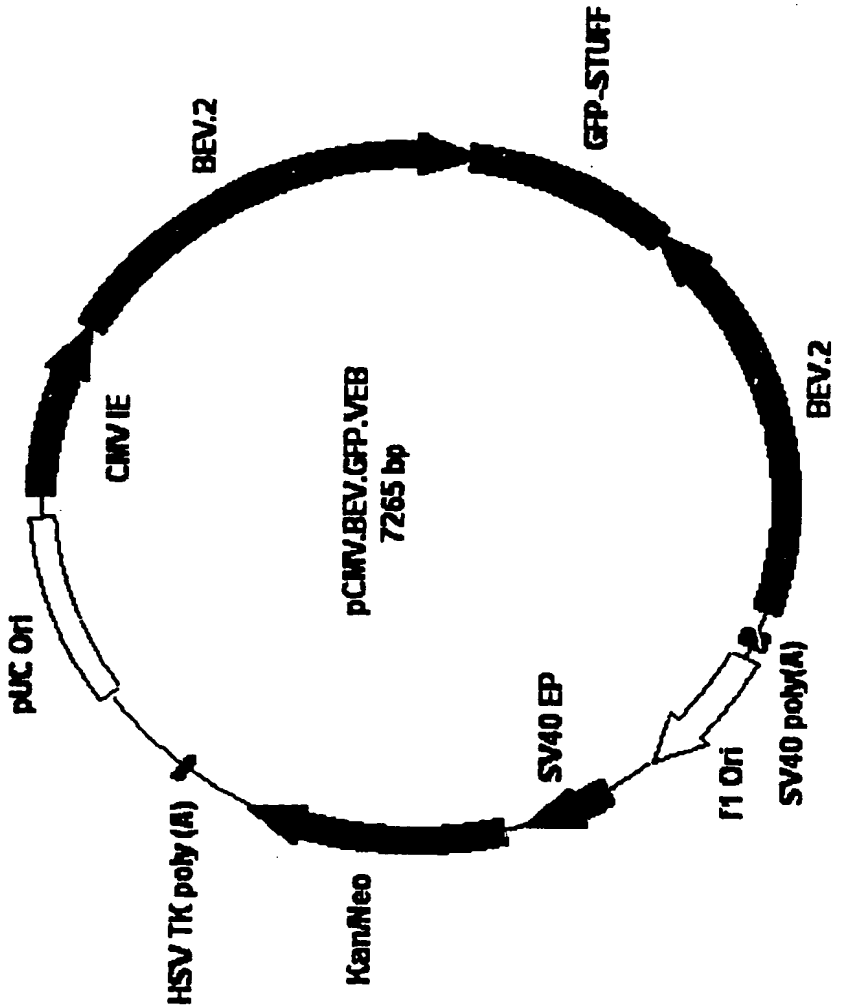
BEV.2
GFP-STUFF
BEV.2
SV40 poly(A)
f1 Ori
SV40 EP
KanNeo
HSV TK poly (A)
pUC Ori
CMV IE
pCMV.BEV.GFP.VEB
7265 bp

FIGURE 7

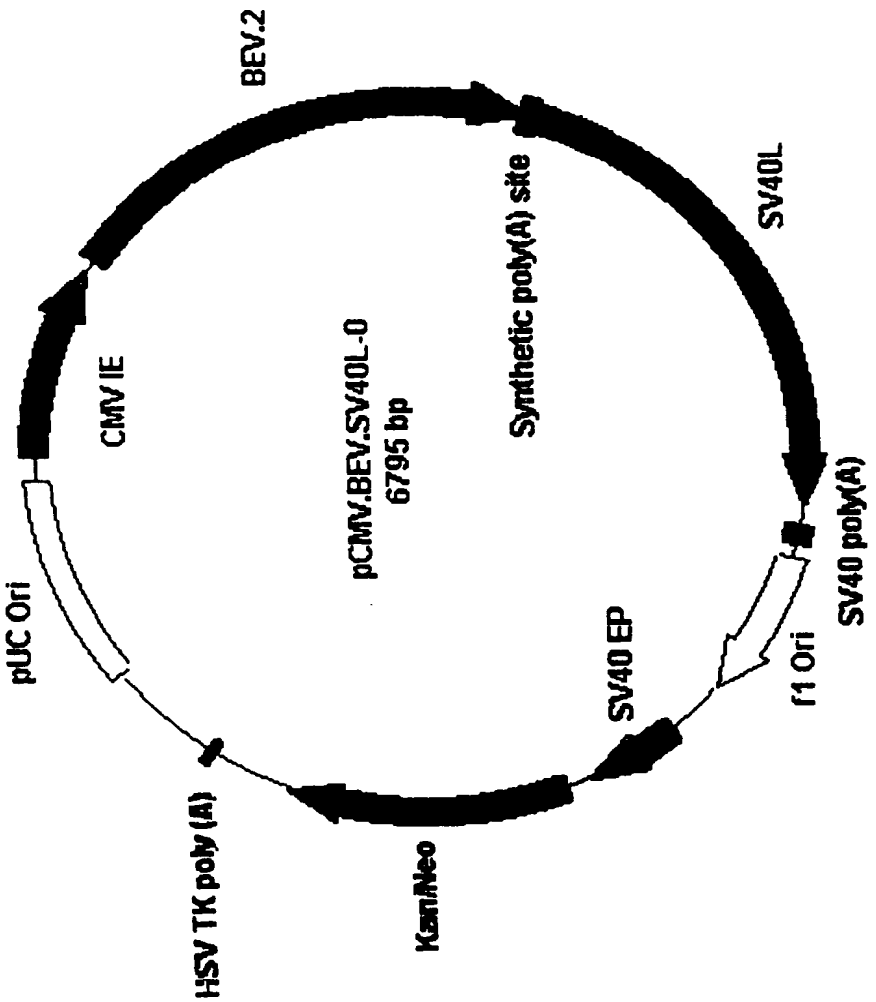
BEV.2
SV40L
Synthetic poly(A) site
CMV IE
pCMV.BEV.SV40L-0
6795 bp
pUC Ori
SV40 poly(A)
f1 Ori
SV40 EP
HSV TK poly (A)
Kan/Neo

FIGURE 8

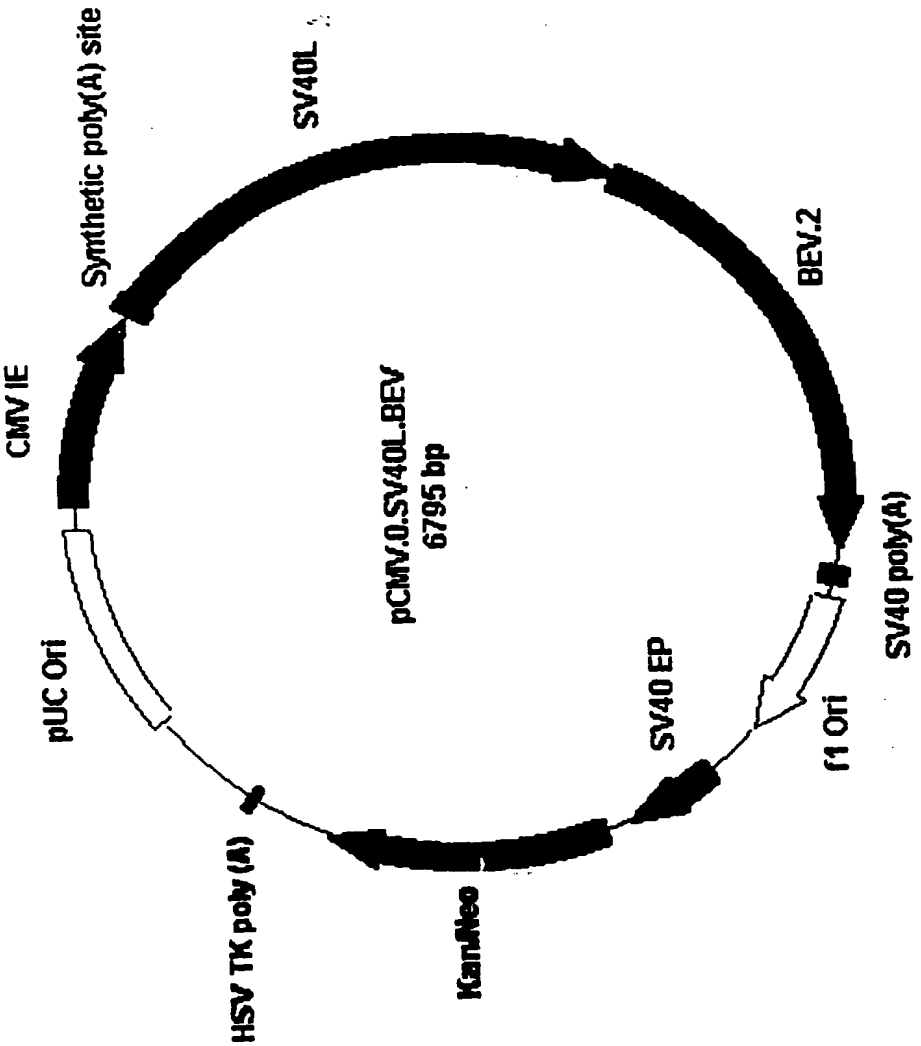
Synthetic poly(A) site
SV40L
BEV.2
CMV IE
pCMV.0.SV40L.BEV
6795 bp
SV40 poly(A)
pUC Ori
SV40 EP
f1 Ori
HSV TK poly (A)
KanNeo

FIGURE 9

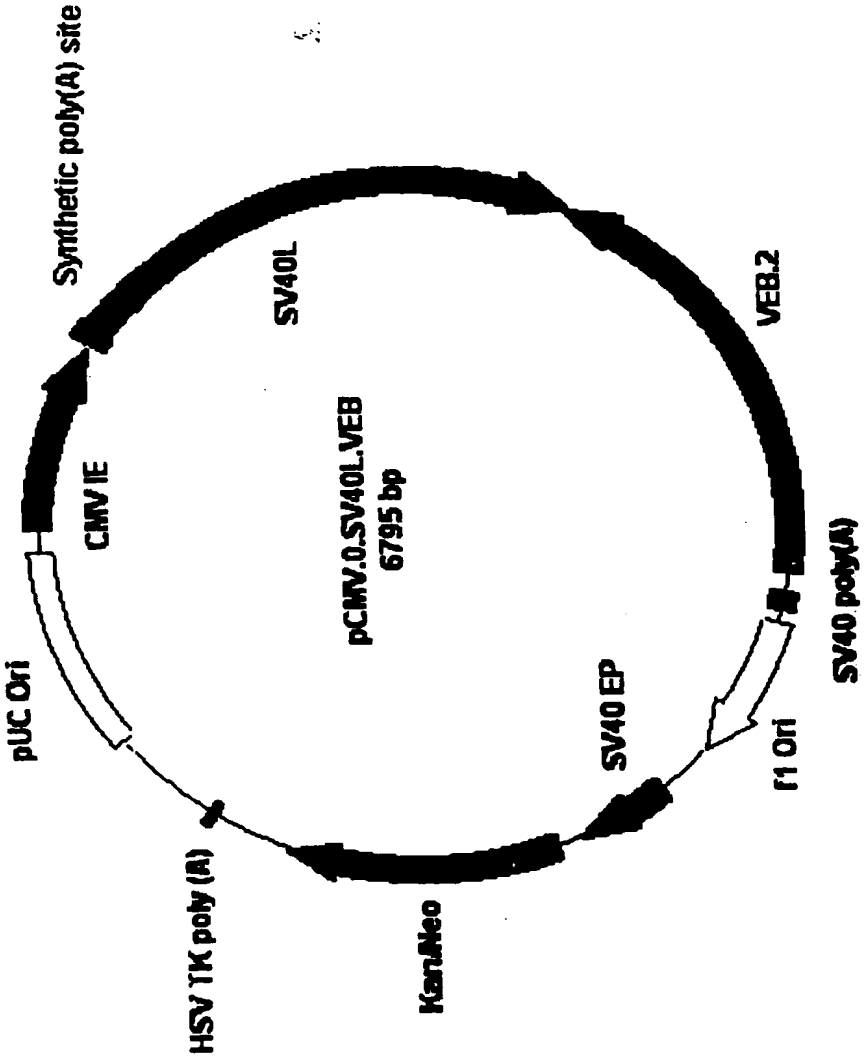
Synthetic poly(A) site
SV40L
VEB.2
CMV IE
pCMV.0.SV40L.VEB
6795 bp
SV40 poly(A)
pUC Ori
SV40 EP
f1 Ori
HSV TK poly (A)
KanNeo

FIGURE 10

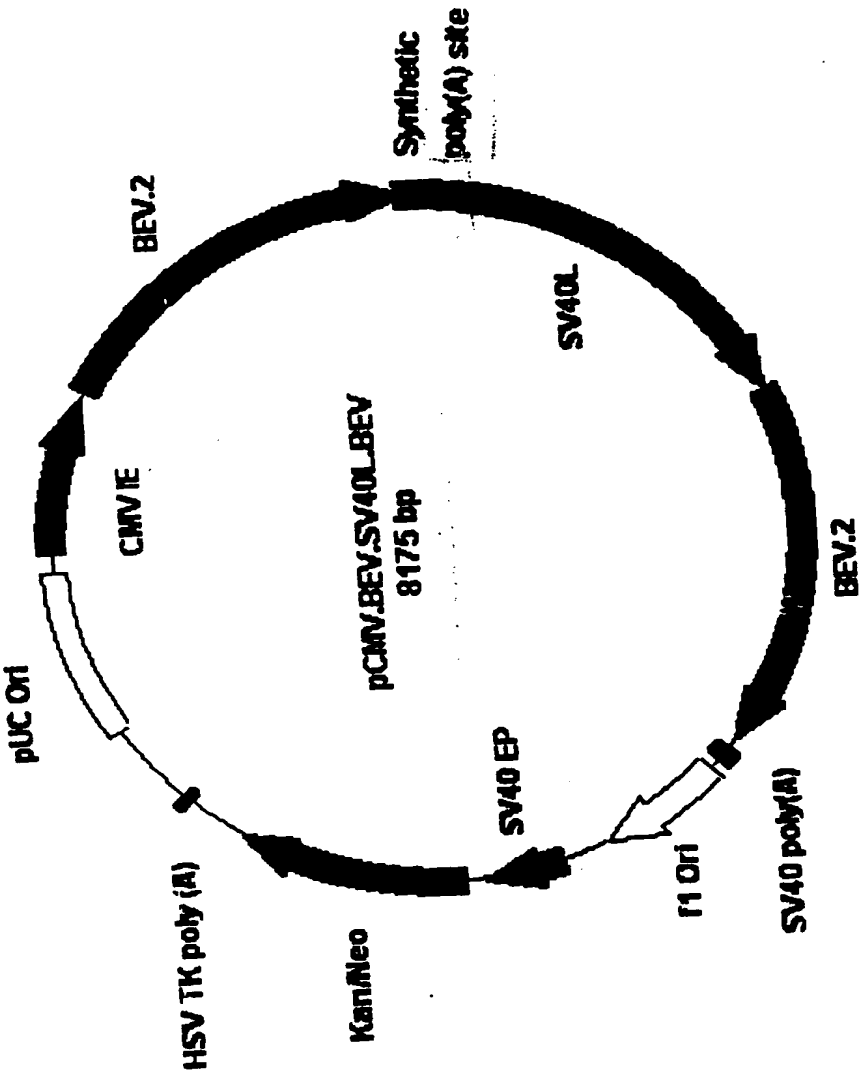
Synthetic poly(A) site
BEV.2
SV40L
pCMV.BEV.SV40L.BEV
8175 bp
CMV IE
BEV.2
pUC Ori
SV40 EP
SV40 poly(A)
f1 Ori
HSV TK poly (A)
Kan/Neo

FIGURE 11

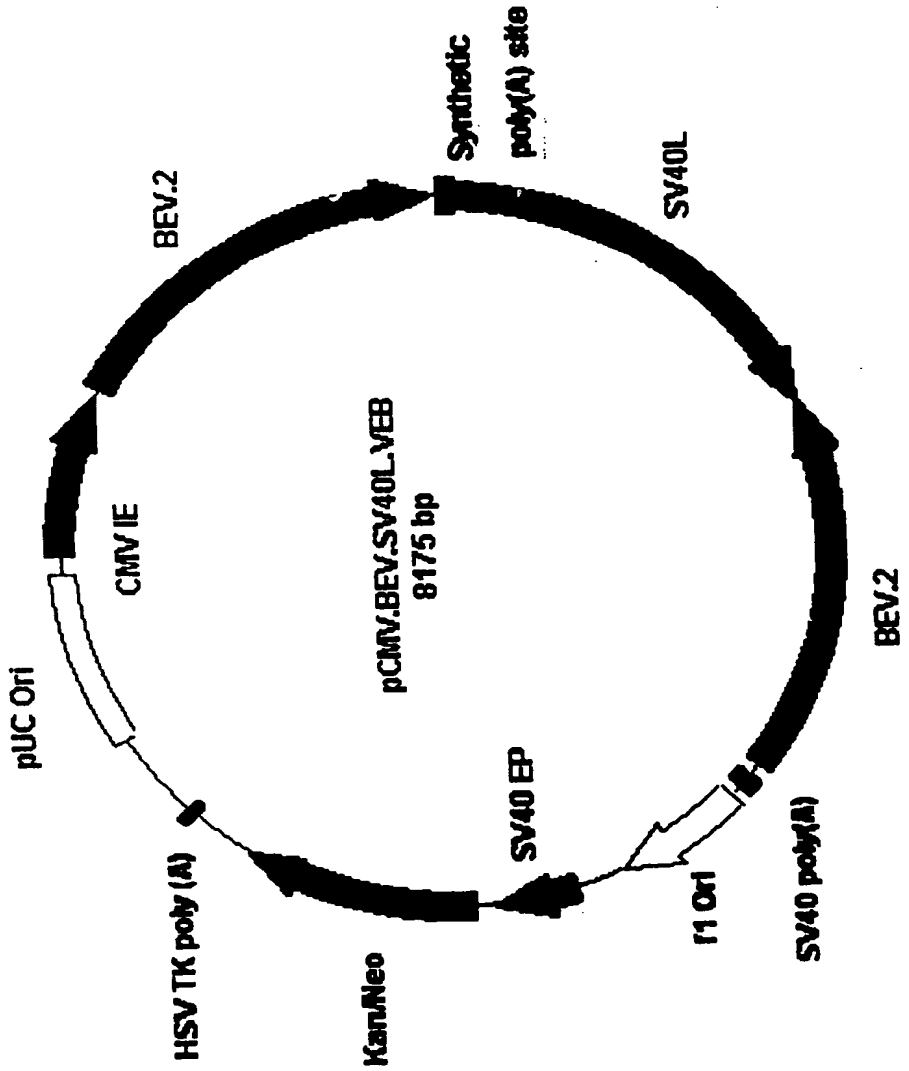
Synthetic
poly(A) site
SV40L
BEV.2
pCMV.BEV.SV40LVEB
8175 bp
CMV IE
pUC Ori
HSV TK poly (A)
Kan/Neo
SV40 EP
f1 Ori
SV40 poly(A)
BEV.2

FIGURE 12

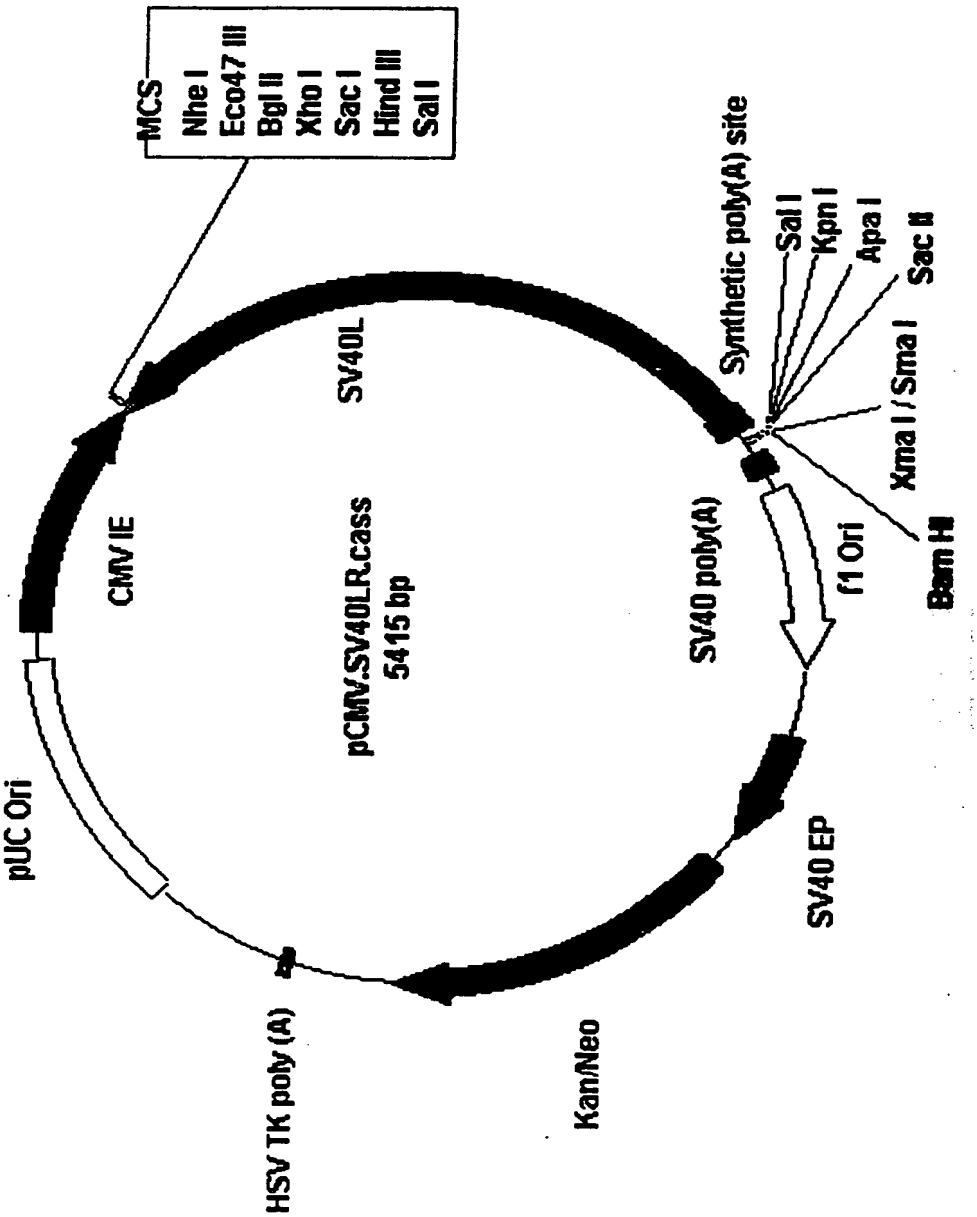
MCS
Nhe I
Eco47 III
Bgl II
Xho I
Sac I
Hind III
Sal I
Synthetic poly(A) site
Sal I
Kpn I
Apa I
Sac II
Xma I / Sma I
BamH I
SV40L
CMV IE
pCMV.SV40LR.cass
5415 bp
SV40 poly(A)
f1 Ori
pUC Ori
SV40 EP
HSV TK poly (A)
Kan/Neo

FIGURE 13

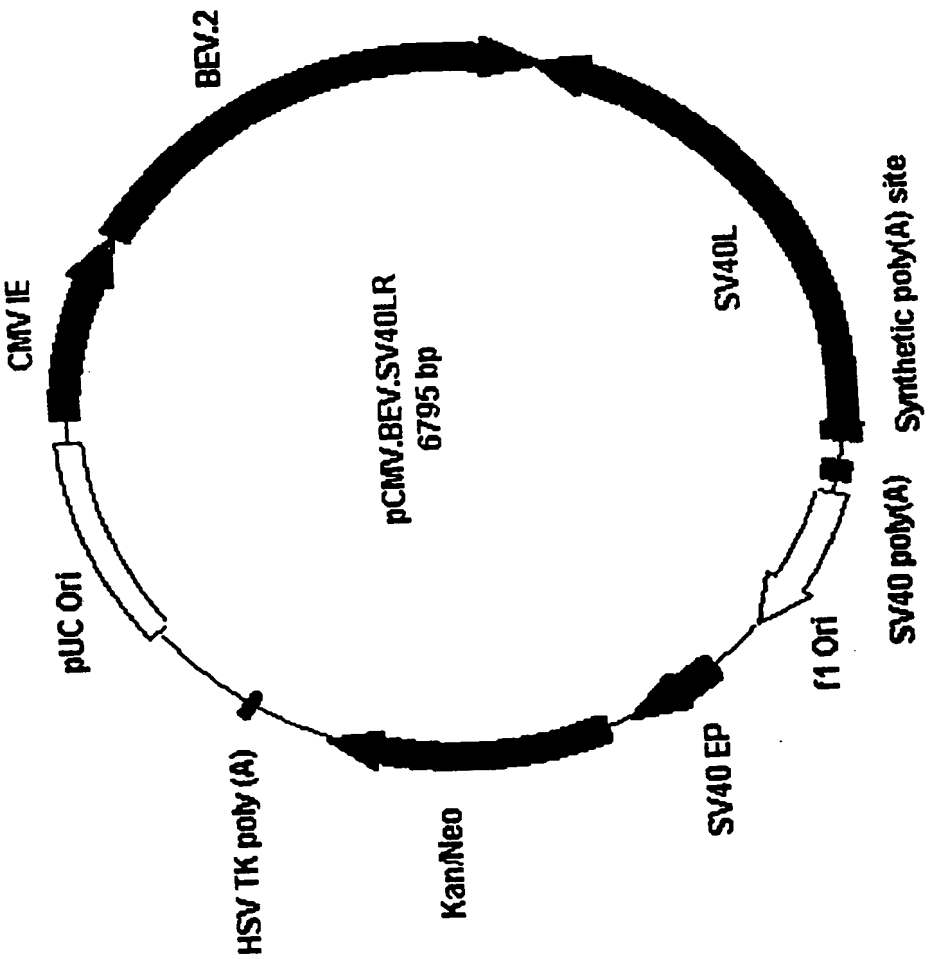
BEV.2
CMV IE
pUC Ori
HSV TK poly (A)
Kan/Neo
SV40 EP
f1 Ori
SV40 poly(A)
Synthetic poly(A) site
SV40L
pCMV.BEV.SV40LR
6795 bp

FIGURE 14

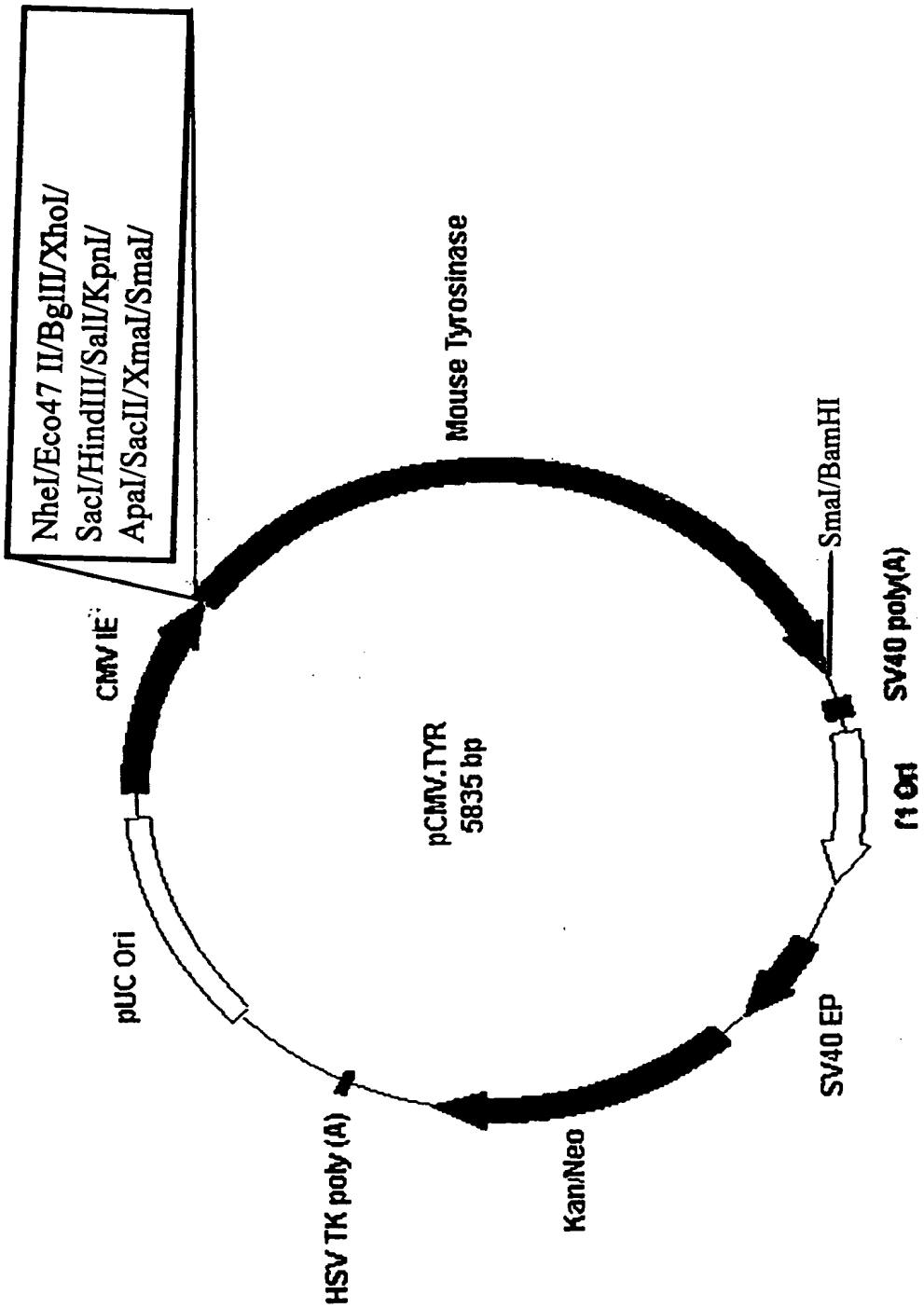
NheI/Eco47 II/BglII/XhoI/
SacI/HindIII/SalI/KpnI/
ApaI/SacII/XmaI/SmaI/
Mouse Tyrosinase
SmaI/BamHI
SV40 poly(A)
f1 Ori
SV40 EP
Kan/Neo
HSV TK poly (A)
pUC Ori
CMV IE
pCMV.TYR
5835 bp

FIGURE 15

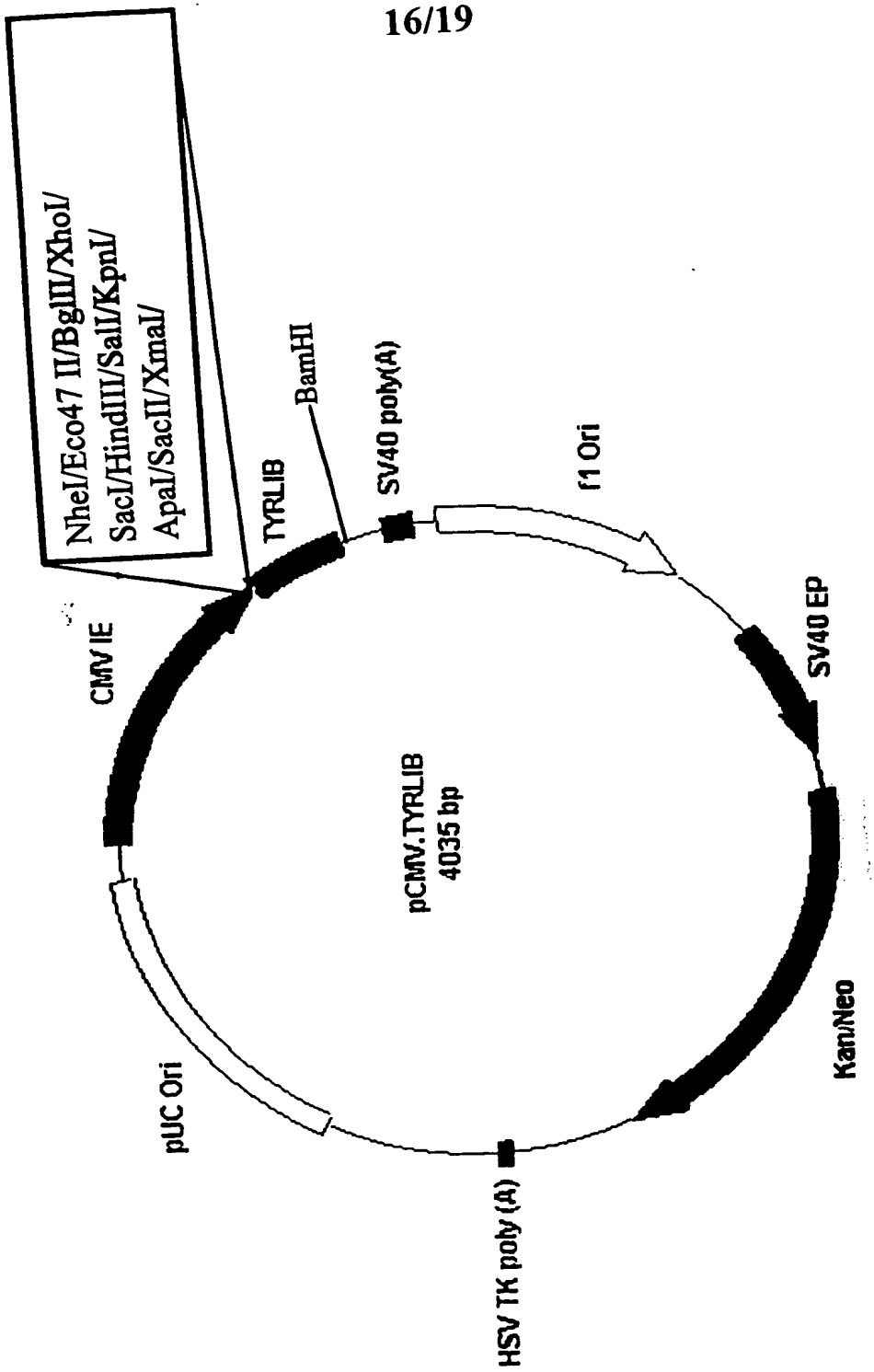
NheI/Eco47 II/BglII/XhoI/
SacI/HindIII/SalI/KpnI/
ApaI/SacII/XmaI/
TYRLIB
BamHI
SV40 poly(A)
f1 Ori
SV40 EP
Kan/Neo
HSV TK poly (A)
pUC Ori
CMV IE
pCMV.TYRLIB
4035 bp

FIGURE 16

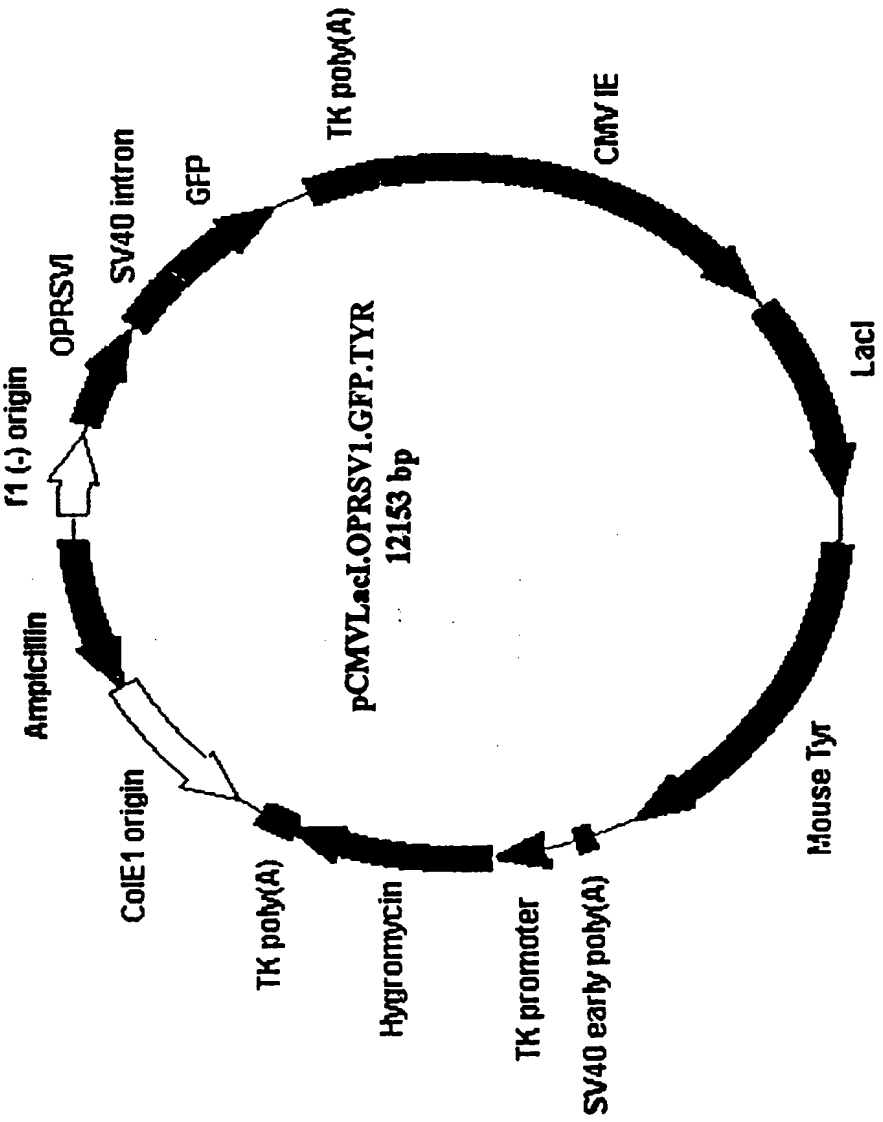
pCMVLacI.OPRSV1.GFP.TYR
12153 bp
TK poly(A)
CMV IE
LacI
Mouse Tyr
SV40 early poly(A)
TK promoter
Hygromycin
TK poly(A)
ColE1 origin
Ampicillin
f1 (-) origin
OPRSVI
SV40 intron
GFP

FIGURE 17

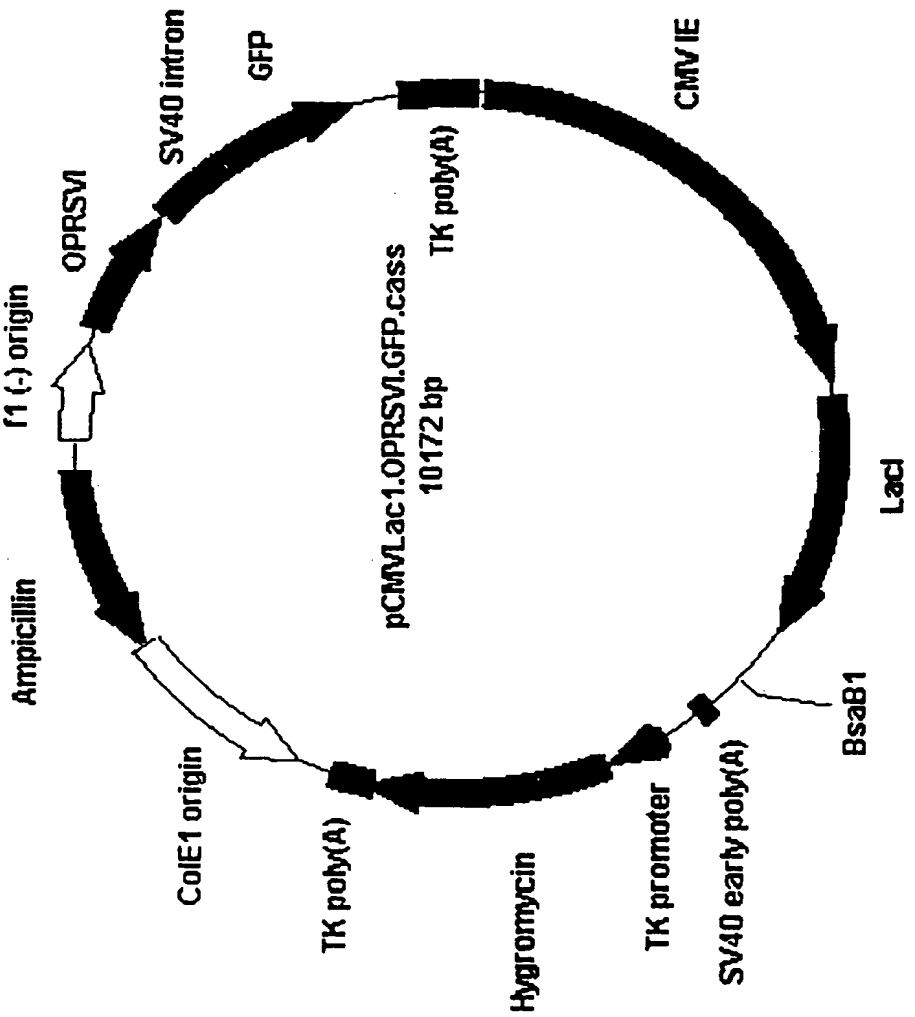
pCMVLac1.OPRSVI.GFP.cass
10172 bp
CMV IE
TK poly(A)
GFP
SV40 intron
OPRSVI
f1 (-) origin
Ampicillin
ColE1 origin
TK poly(A)
Hygromycin
TK promoter
SV40 early poly(A)
BsaB1
LacI

FIGURE 18

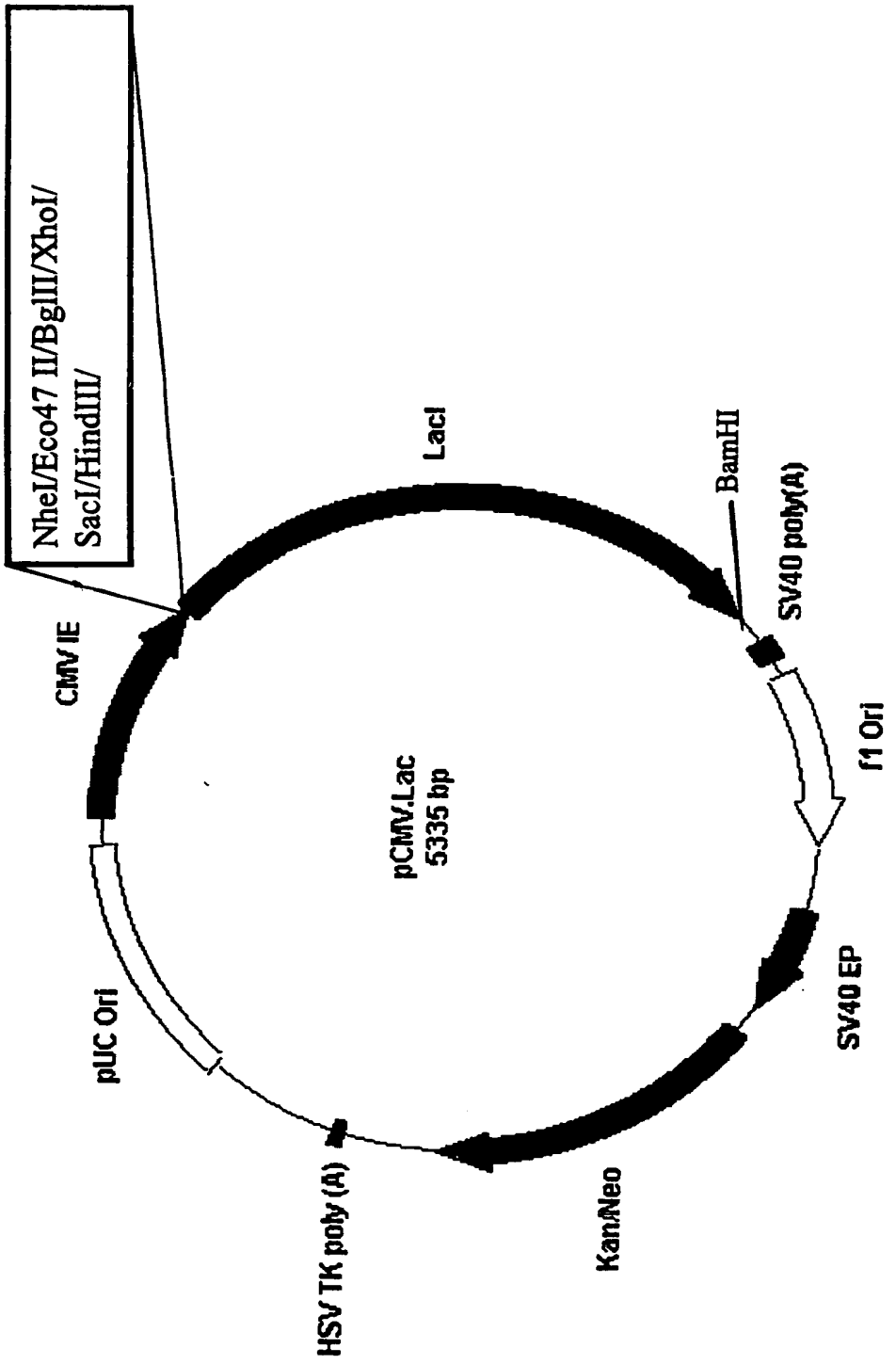
NheI/Eco47 II/BglII/XhoI/
SacI/HindIII/
LacI
BamHI
SV40 poly(A)
f1 Ori
SV40 EP
KanNeo
HSV TK poly (A)
pUC Ori
CMV IE
pCMV.Lac
5335 bp

FIGURE 19